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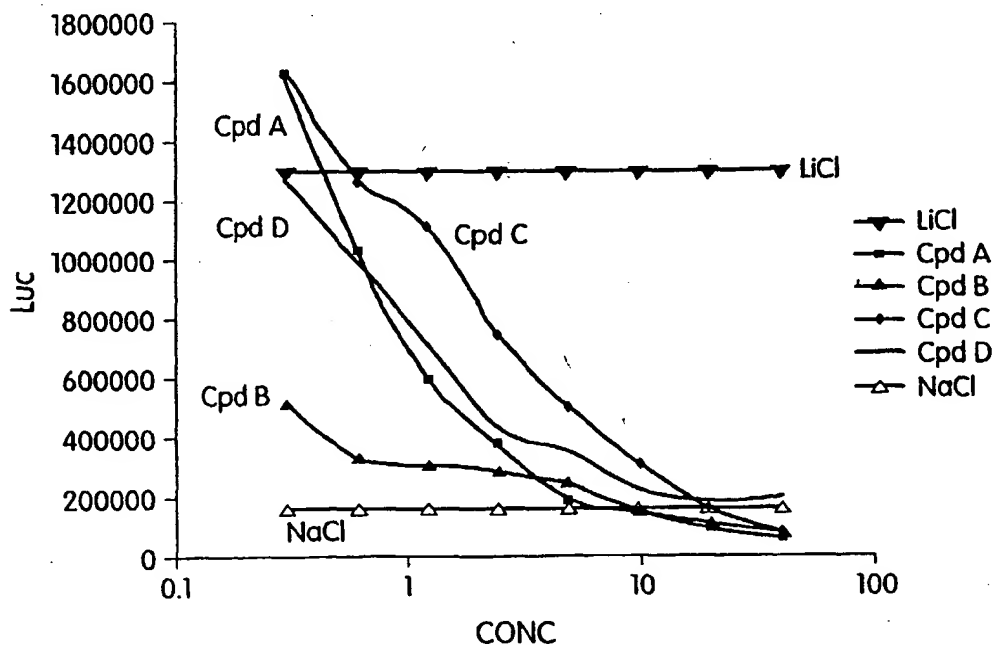
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(54) Title: WNT SIGNALLING ASSAY, METHODS AND USES THEREOF



(57) Abstract: The present invention relates to methods for monitoring the level of activity of the Wnt signaling pathway and provides means to identify factors capable of modulating Wnt signaling. The present invention further concerns nucleic acid constructs, chimeric proteins and cell lines for carrying out the methods of the invention.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WNT SIGNALLING ASSAY, METHODS AND USES THEREOF

Background of the Invention

The Wnt gene family encodes secreted ligand proteins that serve key roles in differentiation and development. This family comprises at least 15 vertebrate and invertebrate genes including the *Drosophila* segment polarity gene *wingless* and one of its vertebrate homologues, *integrated* from which the Wnt name derives. The Wnt proteins appear to facilitate a number of developmental and homeostatic processes. For example, vertebrate *Wnt1* appears to be active in inducing myotome formation within the somites and in establishing the boundaries of the midbrain (see McMahon and Bradley (1990) Cell 62: 1073; Ku and Melton (1993) Development 119: 1161; Stern et al. (1995) Development 121: 3675). During mammalian gastrulation, *Wnt3a*, *Wnt5a*, and *Wnt5b* are expressed in distinct yet overlapping regions within the primitive streak. *Wnt3a* is the only Wnt protein seen in the regions of the streak that will generate the dorsal (somite) mesoderm, and mice homozygous for a null allele of the *Wnt3a* gene have no somites caudal to the forelimbs. The *Wnt* genes also are important in establishing the polarity of vertebrate limbs, just as the invertebrate homolog *wingless* has been shown to establish polarity during insect limb development. In both cases there are interactions with Hedgehog family members as well.

The Wnt signaling pathway comprises a number of proteins involved in the transduction of Wnt/wingless signaling and is intimately connected to the hedgehog developmental pathway. In *Drosophila*, the secreted wingless protein mediates reciprocal interaction between cells in the wingless-hedgehog pathway by binding to neighboring cells through the Frizzled receptor. The Frizzled receptor then activates Disheveled protein, which blocks the inhibiting action of Zeste-white-3 kinase (or GSK-3 beta in vertebrates, Glycogen Synthase Kinase-3 beta) upon the Armadillo protein (a beta-catenin protein). The beta-catenin protein transduces the Wnt signal from the cytoplasm to the nucleus. In the absence of Wnt signaling, beta-catenin is constitutively degraded by the proteasome and can be found in a multimeric complex with conductin (or axin), APC (Adenomatous Polyposis Coli) and GSK-3 beta. APC mediates the binding of beta-catenin to conductin and serves to activate the conductin protein. Conductin acts as a scaffold to assemble the components of

the degradation pathway of beta-catenin. GSK-3 beta, a serine/threonine kinase, phosphorylates beta-catenin thus stimulating its degradation by the proteasome. Upon Wnt signaling, the GSK-3 beta kinase is inactivated leading to stabilization of the beta-catenin protein. beta-Catenin is then released from the multimeric complex
5 and translocates into the nucleus.

Once in the nucleus, beta-catenin interacts with the LEF/TCF (Lymphoid Enhancer Factor/ T-Cell Factor) family of HMG (High Mobility Group) box transcription factors. The LEF/TCF factors are stimulated through interaction with beta-catenin to become potent transactivators of a number of genes including c-myc, cyclin D1, c-jun and *hedgehog* (*hh*). Hedgehog is a secreted protein which can bind
10 to cells adjacent to the Wnt/wingless-activated cell through another receptor, the Patched protein. Binding of the Hedgehog protein to the Patched receptor activates nuclear expression of the wingless protein, which is then secreted and further reinforces the reciprocal signaling with the neighboring hedgehog-secreting cell.

15 Mutations leading to constitutive activation of the Wnt pathway are critical events in a variety of human cancers including colon cancer, melanoma, hepatocellular carcinoma and others. The end result of constitutive activation of the Wnt pathway is a dramatic increase in the level of beta-catenin protein in the cytoplasm. Inappropriate stabilization of beta-catenin, leading to increased levels of
20 the protein, can be caused by mutations in a variety of proteins in the Wnt signaling pathway. For example, stabilizing mutations in beta-catenin (Akiyama (2000) Cytokine Growth Factor Rev. 11: 273), loss of function or dominant negative mutations of GSK-3 beta, mutations in Axin which inhibit GSK-3 beta activity (Hedgepeth et al. (1999) Mol. Cell Biol. 19: 7147) and truncation mutations of APC
25 (Akiyama (2000) Cytokine Growth Factor Rev. 11: 273), have all been linked to increased levels of beta-catenin. Furthermore, it has recently been shown that lithium mimics Wnt signaling through direct inhibition of GSK-3 beta kinase leading to stabilization and accumulation of the beta-catenin protein (Hedgepeth et al. (1997) Dev. Biol. 185: 82; Takahashi et al. (1999) J. Neurochem 73:2073).

30 It is an object of the present invention to provide a method for identifying compounds that can modulate the activity of the Wnt signaling pathway and to provide a means for identifying compounds that are capable of alleviating at least

one of the symptoms associated with certain cancers such as colorectal, desmoid, endometrial, gastric, hepatocellular, hepatoblastoma, kidney (e.g. Wilm's tumor), medulloblastoma, melanoma, ovarian, pancreatic tumors, pilomatricoma, prostate, thyroid, uterine, etc. The invention further provides nucleic acid constructs, chimeric
5 proteins and cell lines for carrying out the methods of the invention.

Summary of the Invention

In one aspect, the present invention relates to a method for modulating or assaying the activity of the Wnt-signaling pathway. In particular, the method comprises studying the interaction of at least two members of the Wnt-signaling
10 pathway, for example, beta-catenin and TCF-4/LEF. In one embodiment, the interaction may be studied by using the two-hybrid system.

In one embodiment, the invention comprises a method for assaying for compounds that may regulate beta-catenin mediated transcription. In particular, the method comprises the use of chimeric genes which express hybrid proteins. To
15 illustrate, the method comprises, transfecting a cell with a first hybrid construct encoding for a DNA binding domain of a transcription factor operably linked to a beta-catenin binding domain. The second hybrid construct comprises a nucleic acid encoding a reporter gene which is located downstream from sequences recognized by the DNA binding domain of the transcription factor. The presence and/or
20 accumulation of beta-catenin in the cell causes the expression of the reporter gene which is operably linked to a transcriptional regulatory site responsive to beta-catenin. The expression of the reporter gene may be detected and measured by means which are within the purview of the skilled artisan.

In another embodiment, the invention comprises a method for identifying a
25 compound, for example, agonists and antagonists capable of affecting Wnt mediated signal transduction. The method comprises transfecting a cell with a first hybrid construct encoding for a DNA binding domain of a transcription factor operably linked to a beta-catenin binding domain. The second hybrid construct comprises a nucleic acid encoding a reporter gene which is located downstream from sequences
30 recognized by the DNA binding domain of the transcription factor. The presence and/or accumulation of beta-catenin in the cell causes the expression of the reporter gene which is operably linked to a transcriptional regulatory site responsive to beta-

catenin. In one aspect, the method comprises inducing beta-catenin accumulation and contacting the cell with a test compound before, after or concurrently with induction of beta-catenin accumulation, and measuring the level of transcription of the gene encoded for by the second nucleic acid construct.

5 In another embodiment, the invention comprises a method for screening for compounds, for example, agonists and antagonists capable of alleviating at least one symptom associated with a cellular proliferative disorder. The method comprises transfecting a cell with a first hybrid construct encoding for a DNA binding domain of a transcription factor operably linked to a beta-catenin binding domain. The
10 second hybrid construct comprises a nucleic acid encoding a reporter gene which is located downstream from sequences recognized by the DNA binding domain of the transcription factor. The presence and/or accumulation of beta-catenin in the cell causes the expression of the reporter gene which is operably linked to a transcriptional regulatory site responsive to beta-catenin. In one aspect, the method
15 comprises inducing beta-catenin accumulation and contacting the cell with a test compound before, after or concurrently with induction of beta-catenin accumulation, and measuring the level of transcription of the gene encoded for by the second nucleic acid construct.

 In another embodiment, the invention comprises a method for affecting Wnt
20 signal transduction. The method involves contacting a cell with an amount of a compound which modulates beta-catenin mediated transcriptional control, effective to change Wnt signal transduction.

 In another embodiment, the invention also provides nucleic acid constructs, chimeric proteins and cell lines for carrying out the methods of the invention.

25 In one embodiment, the invention features a nucleic acid construct encoding for a DNA binding domain of a transcription factor operably linked to a reporter gene.

 In another embodiment, the invention features a transcription factor comprising a DNA binding domain operably linked to a beta-catenin binding
30 domain of factors such as, TCF-4/LEF.

 A further embodiment of the invention features a host cell expressing the DNA binding domain of a transcription factor operably linked to a reporter gene.

Yet another embodiment of the invention features a cultured cell comprising a the DNA binding domain comprising transcription factor operably linked to a beta-catenin binding domain of a transcription factor such as TCF-4/LEF.

In another embodiment, the invention features a host cell comprising a first
5 nucleic acid construct encoding for a transcription factor comprising a DNA binding domain operably linked to a beta-catenin binding domain, and a second nucleic acid construct comprising coding sequences for a reporter gene located downstream from sequences recognized by the DNA binding domain of the transcription factor encoded for by the first nucleic acid construct.

10 In preferred embodiments, the DNA binding domain is derived from a transcription factor, preferably the yeast GAL-4 protein.

In other preferred embodiments, the beta-catenin binding domain is derived from a member of the LEF/TCF family of transcription factors.

In certain preferred embodiments, the expression of the reporter gene may be
15 measured spectroscopically.

In yet other preferred embodiments, beta-catenin accumulation is induced by incubating the transfected cell with a lithium salt.

In other embodiments, preferred cells are human epithelial cells.

In particularly preferred embodiments, the methods of the invention are
20 adaptable to a high throughput format.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for
25 example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames
30 & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology*

(Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987);
5 *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

10 **Detailed Description of the Drawings**

Figure 1. Graph showing the level of luciferase produced in HEK-293 cells in response to an increasing level of lithium chloride (0-100 mM LiCl). The HEK-293 cells were transiently transfected with the luciferase reporter plasmid alone (E1b-luc only), the reporter plasmid plus an expression vector containing a GAL-4 DBD (DNA binding domain) without a catenin binding domain (GAL-DBD), the
15 reporter plasmid plus an expression vector containing a GAL-4 DBD-catenin binding domain (GAL4-LEF) or the reporter plasmid plus an expression vector containing a GAL-4 DBD fused to a fragment of the catenin binding domain lacking most of the catenin binding domain (GAL4-DNLEF).

20 **Figure 2.** Graph showing the level of luciferase produced in TCF2 cells in response to an increasing level of lithium chloride (0-150 mM LiCl). TCF2 cells are HEK-293 cells transfected with a linearized reporter plasmid encoding for luciferase, an effector plasmid encoding for TCF-GAL-4 fusions and a plasmid containing a selectable marker (Zeocin).

25 **Figure 3.** Graph showing the level of luciferase produced in TCF2 cells stimulated with lithium chloride (LiCl) or stimulated with LiCl in the presence of test compounds A-D (Cpd A-D) as compared to unstimulated control cells (NaCl).

Compounds A-D have the following structures:

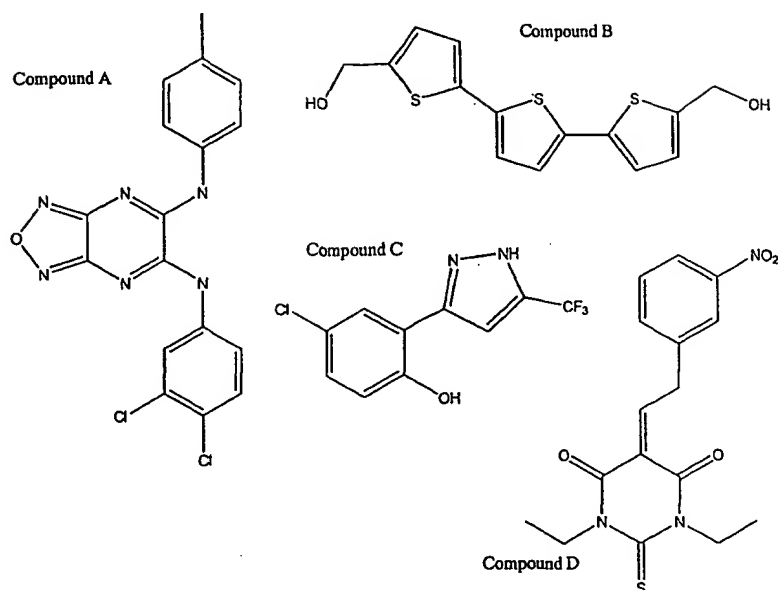


Figure 4. Graph showing the effect of Compound B on SW 480 cells.

Figure 5. Graph showing the effect of Compound B on HepG2 cells.

5 **Best Mode for Carrying Out the Invention**

(i) Overview

Wnt signal transduction is critical to a wide variety of developmental processes including segmentation, CNS patterning and control of asymmetrical cell division. The ultimate target of Wnt signaling is the LEF/TCF family of HMG box
 10 transcription factors. LEF/TCF transcription is stimulated through interaction with a beta-catenin protein. beta-Catenin protein is stabilized in response to Wnt signaling causing it to accumulate in the cytoplasm and then translocate into the nucleus to stimulate transcription of a variety of target genes, including c-myc, c-jun and cyclin D1, among others.

15 Various mutations leading to an increase in beta-catenin levels have been implicated in a number of cancers. Therefore, compounds capable of attenuating beta-catenin mediated transcription would be promising candidates for cancer therapeutics.

Accordingly, the present invention provides a means for assaying beta-
 20 catenin mediated transcription. The invention further provides a means for screening test compounds for the ability to modulate beta-catenin mediated transcription. The

methods of the invention would be particularly useful for high-throughput screening of libraries of test compounds for their effects on beta-catenin mediated transcription. Compounds identified using the methods of the invention would be ideal lead candidates for development of cancer therapeutics.

5 In general the methods of the invention involve transfecting a cell with a gene under the transcriptional control of beta catenin, inducing beta-catenin accumulation and measuring the level of transcription of the gene under the transcriptional control of beta-catenin.

(ii) Definitions

10 For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The term "agonist", as used herein, is meant to refer to an agent that mimics or upregulates (e.g. potentiates or supplements) a Wnt-signaling bioactivity. An agonist can be a wild-type beta-catenin protein or derivative thereof having at least one bioactivity of the wild-type beta-catenin. An Agonist Therapeutic can also be a compound that upregulates expression of a member of the Wnt-signaling pathway such as the beta-catenin gene or which increases at least one bioactivity of the beta-catenin protein. An agonist can also be a compound which increases the interaction of a beta-catenin polypeptide with another molecule, e.g, a member of the TCF-4/LEF family.

20 As used herein the term "animal" refers to mammals, preferably mammals such as humans.

"Antagonist" as used herein is meant to refer to an agent that downregulates (e.g. suppresses or inhibits) at least one Wnt-signaling bioactivity. An "Antagonist Therapeutic" can be a compound which inhibits or decreases the interaction between a beta catenin protein and another molecule, e.g., a member of the TCF-4/LEF family. An antagonist can also be a compound that downregulates expression member of the Wnt-signaling pathway such as the beta-catenin gene or which reduces at least one bioactivity of the beta-catenin protein. An Antagonist Therapeutic can be a dominant negative form of the beta-catenin polypeptide, e.g., a form of the beta-catenin polypeptide which is capable of interacting with a member of the TCF-4/LEF family. The Antagonist Therapeutic can also be a nucleic acid

encoding a dominant negative form of an beta-catenin, a beta-catenin antisense nucleic acid, or a ribozyme capable of interacting specifically with a beta-catenin RNA. Yet other antagonists are molecules which bind to the beta-catenin polypeptide and inhibit its action. Such molecules include peptides, e.g., forms of
5 the beta-catenin peptides which do not have biological activity. Thus, such peptides will bind the active site of beta-catenin and prevent it from interacting with a member of the TCF-4/LEF family. Yet other antagonists include antibodies interacting specifically with an epitope of an beta-catenin molecule, such that binding interferes the interaction of the beta-catenin. In yet another preferred
10 embodiment, the Antagonist Therapeutic is a small molecule, such as a molecule capable of inhibiting the interaction between an beta-catenin polypeptide and a member of the TCF-4/LEF family.

The terms "beta-catenin" or "beta-catenin protein" refer to beta-catenin proteins, or functional equivalents thereof, from any species. A wide variety of beta-
15 catenin proteins from a number of species are known, including, for example, human Plakoglobin (Accession number NP_002221), *Musca domestica* Armadillo (Q02453), mouse beta-catenin (S35091), *Xenopus* beta-catenin (AAA49670), etc.

The term "beta-catenin binding domain" indicates an amino acid sequence which mediates association of a protein with beta-catenin.

20 The phrases "beta-catenin mediated transcriptional activation" or "beta-catenin mediated transcriptional control" indicate that a gene which is under the transcriptional control of the LEF/TCF family of HMG box transcription factors wherein the level of transcript of the gene is changed upon association of a LEF/TCF transcription factor with beta-catenin. The phrase "a gene under the
25 transcriptional control of beta-catenin" means that the level of transcription of the gene is changed in response to a change in the level of beta-catenin protein.

The terms "chimeric" or "chimeric protein" are meant to refer to a protein which is encoded by a nucleic acid that comprises sequences from more than one open reading frame.

30 The terms "compound", "test compound" and "molecule" are used herein interchangeably and are meant to include, but are not limited to, peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries.

The phrase "compound capable of affecting (or modulating) Wnt mediated signal transduction" refers to a compound which alters signal transduction through the Wnt pathway. In general, modulation of Wnt mediated signal transduction will result in a change in the level of beta-catenin protein which will result in a change in the level of transcription of genes under the transcriptional control of the LEF/TCF-beta-catenin complex.

The term "cultured cell" refers to a living cell which is cultivated *in vitro*.

The term "culture medium" is recognized in the art, and refers generally to any substance or preparation used for the cultivation of living cells.

10 "DBD" refers to the DNA binding domain of a protein capable of binding to a specific DNA sequence.

The term "effector plasmid" refers to a vector containing nucleotide sequences encoding for a protein which is capable of affecting the transcription of the reporter gene sequences contained on a reporter plasmid. For example, the effector plasmid can encode for transcription factors or other proteins capable of binding to the promoter/enhancer sequences upstream of the reporter gene on the reporter plasmid and modulating expression of the reporter gene.

The term "GSK-3 beta" refers to GSK-3 beta protein kinases, or functional equivalents thereof, from any species. GSK-3 beta is referred to as Zeste-white-3 kinase (zw3) in flies and as GSK-3 beta (glycogen synthase kinase-3 beta) in vertebrates.

As used herein, "heterologous DNA" or "heterologous nucleic acid" include DNA that does not occur naturally as part of the genome in which it is present or which is found in a location or locations in the genome that differs from that in which it occurs in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Generally, although not necessarily, such DNA encodes RNA and proteins that are not normally produced by the cell in which it is expressed. Heterologous DNA may also be referred to as foreign DNA. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which is expressed is herein encompassed by heterologous DNA. Examples of heterologous DNA include, but are not limited to, DNA that encodes test polypeptides, receptors, reporter genes,

transcriptional and translational regulatory sequences, selectable or traceable marker proteins, such as a protein that confers drug resistance.

The phrase "inducing beta-catenin accumulation" means that the degradation of beta-catenin is inhibited so that the protein is stabilized and its concentration in
5 the cell increases.

The terms "LEF/TCF family of transcription factors" and "LEF/TCF family of HMG box transcription factors" refer to transcription factors from any species capable of binding to HMG box sequences. A wide variety of LEF/TCF transcription factors from a number of species are known, including, for example,
10 human TCF1 (Accession number P36402), mouse TCF1 (Q00417), chicken TCF1, mouse TCF3 (CAA11070), xenopus TCF3, human TCF4 (CAA72166), mouse TCF4 (CAA11071), human LEF-1 (A39625), mouse LEF1 (P27782), chicken LEF1 (AAC24524), Xenopus LEF1, drosophila Pangolin/DTcf (AAC47464), C. elegans Pop-1 (AAC05308), C. elegans Son-1, etc.

15 The term "measured spectroscopically" is meant to indicate measurement of a sample using a variety of techniques including fluorescence detection, UV spectroscopy, scintillation counting, mass spectrometry, IR spectroscopy, etc.

The terms "protein", "polypeptide" and "peptide" are used interchangeably herein.

20 The terms "recombinant protein", "heterologous protein" and "exogenous protein" are used interchangeably throughout the specification and refer to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. That is, the
25 polypeptide is expressed from a heterologous nucleic acid.

The term "reporter plasmid" refers to a vector containing nucleotide sequences encoding for a reporter gene. Reporter genes can be any gene sequence encoding for a protein which provides a means for detecting expression (transcription/translation) of the reporter gene sequences. Reporter genes include,
30 for example, chloramphenicol acetyl transferase, luciferase, beta-galactosidase, alkaline phosphatase, b-lactamase, horseradish peroxidase, green fluorescent protein, glutathione S-transferase, etc. In addition to the reporter gene sequences, the

reporter plasmid may contain promoter/enhancer sequences upstream of the reporter gene which are capable of controlling reporter gene expression.

“Selectable marker” as used herein, refers to the marker and to the nucleic acid encoding said marker. Selectable markers contemplated by the present invention include resistance to antibiotics such as ampicillin, tetracycline, chloramphenicol, kanamycin, neomycin, rifampicin, carbenicillin, streptomycin, Zeocin and the like. The selectable markers also encompass resistance to drugs such as hygromycin and methotrexate, heavy metals such as cadmium, phage infection, and sensitivity to enzymes which affect calorimetric changes such as β -galactosidase.

The term “selection plasmid” refers to a vector containing nucleotide sequences encoding for a selectable marker.

The terms “signal transduction,” “signaling,” “signal transduction pathway,” “signaling pathway,” etc. are used herein interchangeably and refer to the processing of physical or chemical signals from the cellular environment through the cell membrane, and may occur through one or more of several mechanisms, such as activation/inactivation of enzymes (such as proteases, or other enzymes which may alter phosphorylation patterns or other post-translational modifications), activation of ion channels or intracellular ion stores, effector enzyme activation via guanine nucleotide binding protein intermediates, formation of inositol phosphate, activation or inactivation of adenylyl cyclase, direct activation (or inhibition) of a transcriptional factor and/or activation, etc.

“Small molecule” as used herein, is meant to refer to a composition, which has a molecular weight of less than about 5 kD and most preferably less than about 4 kD. Small molecules can be nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic (carbon containing) or inorganic molecules. Many pharmaceutical companies have extensive libraries of chemical and/or biological mixtures, often fungal, bacterial, or algal extracts, which can be screened with any of the assays of the invention to identify compounds that modulate an Wnt bioactivity.

“Wnt function” refers to the activity of the Wnt signalling pathway.

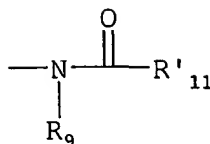
“Wnt pathway” and “Wnt signaling pathway” are used herein interchangeably and refer to the pathway by which binding of the Wnt protein to its extracellular receptor is translated into the nucleus and results in transcriptional activation of a variety of genes. The Wnt signaling pathway involves a variety of
5 proteins including Frizzled, Disheveled, Axin, APC, GSK-3 beta, beta-catenin, LEF/TCF transcription factors, etc. Cells from many different species express homologs of the proteins involved in the Wnt signaling pathway and accordingly have functionally equivalent Wnt signaling pathways.

“Wnt signaling” and “Wnt signal transduction” are used herein
10 interchangeably and refer to the transduction of the signal of Wnt binding to its extracellular receptor into the nucleus resulting in transcriptional activation of a number of genes. Wnt signaling occurs through the Wnt signal transduction pathway.

The term “Wnt therapeutic” refers to various forms of polypeptides such as
15 for example various forms of beta-catenin, as well as peptidomimetics, nucleic acids, or small molecules, which can modulate at least one activity of a member of the Wnt-signaling pathway, e.g., interaction between beta-catenin and a member of the TCF-4/LEF family, by mimicking or potentiating (agonizing) or inhibiting (antagonizing) the effects of a naturally-occurring member of the Wnt-signaling
20 pathway, for example, the beta-catenin polypeptide. In particular, a Wnt therapeutic which mimics or potentiates the activity of a wild-type beta-catenin polypeptide is a “ Agonist Therapeutic”. Conversely, an Wnt therapeutic which inhibits the activity of a wild-type beta-catenin polypeptide is a “ Antagonist Therapeutic”.

The terms “vector” or “plasmid” are used herein interchangeably and refer to
25 a linear or circular nucleotide sequence containing all of the elements necessary for replicating in a cell and for expressing a protein from a nucleotide sequence. Such elements may include, for example, promoter/enhancer sequences, origin or replication, termination sequences, etc. Various plasmid sequences and methods for constructing them are well known to the skilled in the art.

30 The term “acylamino” is art-recognized and refers to a moiety that can be represented by the general formula:



wherein R_9 is as defined above, and R'_{11} represents a hydrogen, an alkyl, an alkenyl or $-(\text{CH}_2)_m\text{-R}_8$, where m and R_8 are as defined above.

Herein, the term "aliphatic group" refers to a straight-chain, branched-chain, or cyclic aliphatic hydrocarbon group and includes saturated and unsaturated aliphatic groups, such as an alkyl group, an alkenyl group, and an alkynyl group.

The terms "alkenyl" and "alkynyl" refer to unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively.

The terms "alkoxyl" or "alkoxy" as used herein refers to an alkyl group, as defined above, having an oxygen radical attached thereto. Representative alkoxyl groups include methoxy, ethoxy, propyloxy, tert-butoxy and the like. An "ether" is two hydrocarbons covalently linked by an oxygen. Accordingly, the substituent of an alkyl that renders that alkyl an ether is or resembles an alkoxyl, such as can be represented by one of $-\text{O-alkyl}$, $-\text{O-alkenyl}$, $-\text{O-alkynyl}$, $-\text{O}-(\text{CH}_2)_m\text{-R}_8$, where m and R_8 are described above.

The term "alkyl" refers to the radical of saturated aliphatic groups, including straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl-substituted cycloalkyl groups, and cycloalkyl-substituted alkyl groups. In preferred embodiments, a straight chain or branched chain alkyl has 30 or fewer carbon atoms in its backbone (e.g., $\text{C}_1\text{-C}_{30}$ for straight chains, $\text{C}_3\text{-C}_{30}$ for branched chains), and more preferably 20 or fewer. Likewise, preferred cycloalkyls have from 3-10 carbon atoms in their ring structure, and more preferably have 5, 6 or 7 carbons in the ring structure.

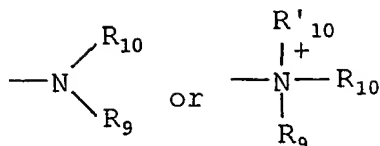
Moreover, the term "alkyl" (or "lower alkyl") as used throughout the specification, examples, and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, a halogen, a hydroxyl, a

carbonyl (such as a carboxyl, an alkoxycarbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxyl, a phosphoryl, a phosphate, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulfhydryl, an alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include substituted and unsubstituted forms of amino, azido, imino, amido, phosphoryl (including phosphonate and phosphinate), sulfonyl (including sulfate, sulfonamido, sulfamoyl and sulfonate), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), -CF₃, -CN and the like. Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with alkyls, alkenyls, alkoxys, alkylthios, aminoalkyls, carbonyl-substituted alkyls, -CF₃, -CN, and the like.

Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, but having from one to ten carbons, more preferably from one to six carbon atoms in its backbone structure. Likewise, "lower alkenyl" and "lower alkynyl" have similar chain lengths. Throughout the application, preferred alkyl groups are lower alkyls. In preferred embodiments, a substituent designated herein as alkyl is a lower alkyl.

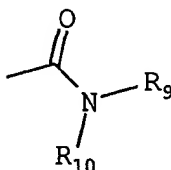
The term "alkylthio" refers to an alkyl group, as defined above, having a sulfur radical attached thereto. In preferred embodiments, the "alkylthio" moiety is represented by one of -S-alkyl, -S-alkenyl, -S-alkynyl, and -S-(CH₂)_m-R_g, wherein m and R_g are defined above. Representative alkylthio groups include methylthio, ethylthio, and the like.

The terms "amine" and "amino" are art-recognized and refer to both unsubstituted and substituted amines, e.g., a moiety that can be represented by the general formula:



wherein R₉, R₁₀ and R'₁₀ each independently represent a hydrogen, an alkyl, an alkenyl, -(CH₂)_m-R₈, or R₉ and R₁₀ taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure;
 5 R₈ represents an aryl, a cycloalkyl, a cycloalkenyl, a heterocycle or a polycycle; and m is zero or an integer in the range of 1 to 8. In preferred embodiments, only one of R₉ or R₁₀ can be a carbonyl, e.g., R₉, R₁₀ and the nitrogen together do not form an imide. In even more preferred embodiments, R₉ and R₁₀ (and optionally R'₁₀) each independently represent a hydrogen, an alkyl, an alkenyl, or -(CH₂)_m-R₈. Thus, the
 10 term "alkylamine" as used herein means an amine group, as defined above, having a substituted or unsubstituted alkyl attached thereto, i.e., at least one of R₉ and R₁₀ is an alkyl group.

The term "amido" is art-recognized as an amino-substituted carbonyl and includes a moiety that can be represented by the general formula:



15

wherein R₉, R₁₀ are as defined above. Preferred embodiments of the amide will not include imides which may be unstable.

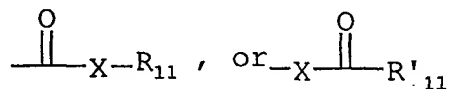
The term "aralkyl", as used herein, refers to an alkyl group substituted with an aryl group (e.g., an aromatic or heteroaromatic group).

20 The term "aryl" as used herein includes 5-, 6-, and 7-membered single-ring aromatic groups that may include from zero to four heteroatoms, for example, benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine, and the like. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl
 25 heterocycles" or "heteroaromatics." The aromatic ring can be substituted at one or

more ring positions with such substituents as described above, for example, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, alkoxyl, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, sulfonamido, ketone, aldehyde, ester, heterocyclyl, aromatic or heteroaromatic moieties, -CF₃, -CN, or the like. The term "aryl" also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings (the rings are "fused rings") wherein at least one of the rings is aromatic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls.

The term "carbocycle", as used herein, refers to an aromatic or non-aromatic ring in which each atom of the ring is carbon.

The term "carbonyl" is art-recognized and includes such moieties as can be represented by the general formula:



wherein X is a bond or represents an oxygen or a sulfur, and R₁₁ represents a hydrogen, an alkyl, an alkenyl, -(CH₂)_m-R₈ or a pharmaceutically acceptable salt, R'₁₁ represents a hydrogen, an alkyl, an alkenyl or -(CH₂)_m-R₈, where m and R₈ are as defined above. Where X is an oxygen and R₁₁ or R'₁₁ is not hydrogen, the formula represents an "ester". Where X is an oxygen, and R₁₁ is as defined above, the moiety is referred to herein as a carboxyl group, and particularly when R₁₁ is a hydrogen, the formula represents a "carboxylic acid". Where X is an oxygen, and R'₁₁ is hydrogen, the formula represents a "formate". In general, where the oxygen atom of the above formula is replaced by sulfur, the formula represents a "thiocarbonyl" group. Where X is a sulfur and R₁₁ or R'₁₁ is not hydrogen, the formula represents a "thioester." Where X is a sulfur and R₁₁ is hydrogen, the formula represents a "thiocarboxylic acid." Where X is a sulfur and R'₁₁ is hydrogen, the formula represents a "thiolformate." On the other hand, where X is a bond, and R₁₁ is not hydrogen, the above formula represents a "ketone" group.

Where X is a bond, and R₁₁ is hydrogen, the above formula represents an "aldehyde" group.

The term "heteroatom" as used herein means an atom of any element other than carbon or hydrogen. Preferred heteroatoms are boron, nitrogen, oxygen, phosphorus, sulfur and selenium.

The terms "heterocyclyl" or "heterocyclic group" refer to 3- to 10-membered ring structures, more preferably 3- to 7-membered rings, whose ring structures include one to four heteroatoms. Heterocycles can also be polycycles. Heterocyclyl groups include, for example, thiophene, thianthrene, furan, pyran, isobenzofuran, chromene, xanthene, phenoxathiin, pyrrole, imidazole, pyrazole, isothiazole, isoxazole, pyridine, pyrazine, pyrimidine, pyridazine, indolizine, isoindole, indole, indazole, purine, quinolizine, isoquinoline, quinoline, phthalazine, naphthyridine, quinoxaline, quinazoline, cinnoline, pteridine, carbazole, carboline, phenanthridine, acridine, pyrimidine, phenanthroline, phenazine, phenarsazine, phenothiazine, furazan, phenoxazine, pyrrolidine, oxolane, thiolane, oxazole, piperidine, piperazine, morpholine, lactones, lactams such as azetidinones and pyrrolidinones, sultams, sultones, and the like. The heterocyclic ring can be substituted at one or more positions with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, -CF₃, -CN, or the like.

As used herein, the term "nitro" means -NO₂; the term "halogen" designates -F, -Cl, -Br or -I; the term "sulfhydryl" means -SH; the term "hydroxyl" means -OH; and the term "sulfonyl" means -SO₂-.

The terms "polycyclyl" or "polycyclic group" refer to two or more rings (e.g., cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls) in which two or more carbons are common to two adjoining rings, e.g., the rings are "fused rings". Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each of the rings of the polycycle can be substituted with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl,

cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, -CF₃, -CN, or the like.

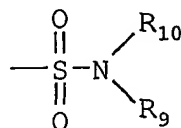
5 The phrase "protecting group" as used herein means temporary substituents which protect a potentially reactive functional group from undesired chemical transformations. Examples of such protecting groups include esters of carboxylic acids, silyl ethers of alcohols, and acetals and ketals of aldehydes and ketones, respectively. The field of protecting group chemistry has been reviewed (Greene,
10 T.W.; Wuts, P.G.M. *Protective Groups in Organic Synthesis*, 2nd ed.; Wiley: New York, 1991).

A "selenoalkyl" refers to an alkyl group having a substituted seleno group attached thereto. Exemplary "selenoethers" which may be substituted on the alkyl are selected from one of -Se-alkyl, -Se-alkenyl, -Se-alkynyl, and -Se-(CH₂)_m-R_g, m
15 and R_g being defined above.

As used herein, the term "substituted" is contemplated to include all permissible substituents of organic compounds. In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic substituents of organic compounds.
20 Illustrative substituents include, for example, those described herein above. The permissible substituents can be one or more and the same or different for appropriate organic compounds. For purposes of this invention, the heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valences of the
25 heteroatoms. This invention is not intended to be limited in any manner by the permissible substituents of organic compounds.

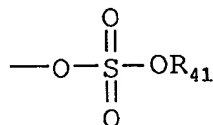
It will be understood that "substitution" or "substituted with" includes the implicit proviso that such substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable
30 compound, e.g., which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc.

The term "sulfamoyl" is art-recognized and includes a moiety that can be represented by the general formula:



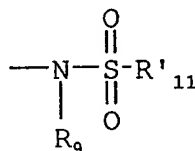
in which R₉ and R₁₀ are as defined above.

5 The term "sulfate" is art recognized and includes a moiety that can be represented by the general formula:



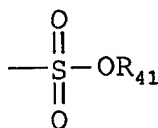
in which R₄₁ is as defined above.

10 The term "sulfonamido" is art recognized and includes a moiety that can be represented by the general formula:



in which R₉ and R'₁₁ are as defined above.

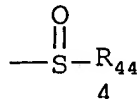
The term "sulfonate" is art-recognized and includes a moiety that can be represented by the general formula:



15

in which R₄₁ is an electron pair, hydrogen, alkyl, cycloalkyl, or aryl.

The terms "sulfoxido" or "sulfinyl", as used herein, refers to a moiety that can be represented by the general formula:



20 in which R₄₄ is selected from hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aralkyl, or aryl.

Analogous substitutions can be made to alkenyl and alkynyl groups to produce, for example, aminoalkenyls, aminoalkynyls, amidoalkenyls, amidoalkynyls, iminoalkenyls, iminoalkynyls, thioalkenyls, thioalkynyls, carbonyl-substituted alkenyls or alkynyls.

5 As used herein, the definition of each expression, e.g., alkyl, m, n, etc., when it occurs more than once in any structure, is intended to be independent of its definition elsewhere in the same structure.

The terms triflyl, tosyl, mesyl, and nonaflyl are art-recognized and refer to trifluoromethanesulfonyl, *p*-toluenesulfonyl, methanesulfonyl, and
10 nonafluorobutanesulfonyl groups, respectively. The terms triflate, tosylate, mesylate, and nonaflate are art-recognized and refer to trifluoromethanesulfonate ester, *p*-toluenesulfonate ester, methanesulfonate ester, and nonafluorobutanesulfonate ester functional groups and molecules that contain said groups, respectively.

15 The abbreviations Me, Et, Ph, Tf, Nf, Ts, Ms represent methyl, ethyl, phenyl, trifluoromethanesulfonyl, nonafluorobutanesulfonyl, *p*-toluenesulfonyl and methanesulfonyl, respectively. A more comprehensive list of the abbreviations utilized by organic chemists of ordinary skill in the art appears in the first issue of each volume of the *Journal of Organic Chemistry*; this list is typically presented in a
20 table entitled Standard List of Abbreviations. The abbreviations contained in said list, and all abbreviations utilized by organic chemists of ordinary skill in the art are hereby incorporated by reference.

Certain compounds of the present invention may exist in particular geometric or stereoisomeric forms. The present invention contemplates all such
25 compounds, including cis- and trans-isomers, *R*- and *S*-enantiomers, diastereomers, (D)-isomers, (L)-isomers, the racemic mixtures thereof, and other mixtures thereof, as falling within the scope of the invention. Additional asymmetric carbon atoms may be present in a substituent such as an alkyl group. All such isomers, as well as mixtures thereof, are intended to be included in this invention.

30 If, for instance, a particular enantiomer of a compound of the present invention is desired, it may be prepared by asymmetric synthesis, or by derivation with a chiral auxiliary, where the resulting diastereomeric mixture is separated and

the auxiliary group cleaved to provide the pure desired enantiomers. Alternatively, where the molecule contains a basic functional group, such as amino, or an acidic functional group, such as carboxyl, diastereomeric salts may be formed with an appropriate optically active acid or base, followed by resolution of the diastereomers thus formed by fractional crystallization or chromatographic means well known in the art, and subsequent recovery of the pure enantiomers.

Contemplated equivalents of the compounds described above include compounds which otherwise correspond thereto, and which have the same general properties thereof (e.g., the ability to inhibit Wnt signaling), wherein one or more simple variations of substituents are made which do not adversely affect the efficacy of the compound. In general, the compounds of the present invention may be prepared by the methods illustrated in the general reaction schemes as, for example, described below, or by modifications thereof, using readily available starting materials, reagents and conventional synthesis procedures. In these reactions, it is also possible to make use of variants which are in themselves known, but are not mentioned here.

For purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics, 67th Ed., 1986-87, inside cover. Also for purposes of this invention, the term "hydrocarbon" is contemplated to include all permissible compounds having at least one hydrogen and one carbon atom. In a broad aspect, the permissible hydrocarbons include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic organic compounds which can be substituted or unsubstituted.

25 (iii) Exemplary Embodiments

Certain terms being set out above, it is noted that one aspect of the present invention features a method for assaying beta-catenin mediated transcription. The method involves transfecting a cell with a gene under the transcriptional control of beta catenin, inducing beta-catenin accumulation and measuring the level of transcription of the gene under the transcriptional control of beta-catenin.

Any cell type may be used, including for example, prokaryotic, yeast, plant, insect, worm, frog, fly, fish, mouse, rat, monkey, animal, mammalian, etc. cells.

Preferably the cells have a naturally occurring Wnt signal transduction pathway. If the cells do not have a Wnt signaling pathway, the cells may be transfected with sequences encoding for the members of the Wnt signaling pathway necessary for achieving the method of the invention. Preferably the cells are capable of being
5 propagated in vitro. Preferred cells of the invention are epithelial cells, more preferably, human epithelial cells, particularly HEK-293 cells (human embryonal kidney cells, ATCC CRL-1573).

Any one of a variety of methods for stably or transiently transfecting a nucleotide sequence into a cell can be used to introduce the gene under the
10 transcriptional control of beta-catenin into the cell. Such transfection methods are well known to the skilled in the art and include, for example, electroporation, calcium phosphate coprecipitation, lipofectin, etc.

The gene under transcriptional control of beta-catenin may be any gene either naturally under the control of beta-catenin or not naturally under the control
15 of beta-catenin transcriptional control. Preferred genes are those that can provide a selectable or screenable trait upon transcriptional activation (or inactivation) in response to induction of beta-catenin. Selectable markers contemplated by the present invention include resistance to antibiotics, drugs, heavy metals, phage infection, etc. Particularly preferred are reporter genes that generate an easily
20 detectable signal. Examples of reporter genes include, but are not limited to, CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), *Nature* 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), *Mol. Cell. Biol.* 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), *PNAS* 1: 4154-4158; Baldwin et al.
25 (1984), *Biochemistry* 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) *Eur. J. Biochem.* 182: 231-238, Hall et al. (1983) *J. Mol. Appl. Gen.* 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) *Methods in Enzymol.* 216:362-368); β -lactamase or GST.

The gene under the transcriptional control of beta-catenin is cloned
30 downstream of nucleotide sequences which provide binding sites for proteins capable of inducing transcription upon binding to beta-catenin. For example, the gene may be cloned downstream from sequences which bind transcription factors

that naturally bind to beta-catenin, for example, HMG box binding sites for LEF/TCF transcription factors. Alternatively, the gene may be cloned downstream from binding sites for transcription factors that do not naturally bind to beta-catenin. In this case, the cell must also be engineered to express a chimeric transcription
5 factor comprising an appropriate DNA binding domain fused to a beta-catenin binding domain.

A wide variety of suitable proteins capable of binding a specific nucleotide sequence are known to those skilled in the art. The entire DNA binding protein, or a fragment thereof which effectively binds DNA, may be fused to the beta-catenin
10 binding domain. Preferred DNA binding domains are derived from transcription factors. An exemplary transcription factor is GAL-4, in particular amino acids 1-147 of GAL-4 which comprises the DNA binding domain of the protein.

The beta-catenin binding domain may be a protein, protein fragment, peptide, synthetic peptide, etc. which is capable of causing an association between a
15 protein and beta-catenin. Beta-catenin binding domain amino acid sequences may be derived from naturally occurring sequences (e.g., sequences contained within a polypeptide encoded for by genomic DNA) or non-naturally occurring sequences (e.g., sequences not found in polypeptides encoded for by genomic DNA). Determination of beta-catenin binding domain sequences may be carried out by any
20 method known in the art, such as, isolation of beta-catenin binding peptides from a phage display library, affinity purification of peptide sequences from a library using beta-catenin as a bait protein, two-hybrid assay using beta-catenin as a bait protein, etc. Preferred examples of beta-catenin binding domains are derived from proteins containing beta-catenin interaction domains including, but not limited to, the
25 LEF/TCF family of transcription factors. A wide variety of LEF/TCF transcription factors from a number of species are known, including, for example, human TCF1 (Accession number P36402), mouse TCF1 (Q00417), chicken TCF1, mouse TCF3 (CAA11070), xenopus TCF3, human TCF4 (CAA72166), mouse TCF4 (CAA11071), human LEF-1 (A39625), mouse LEF1 (P27782), chicken LEF1
30 (AAC24524), Xenopus LEF1 (AF063831), drosophila Pangolin/DTcf (AAC47464), C. elegans Pop-1 (AAC05308), C. elegans Son-1, etc. Amino terminal fragments of LEF/TCF transcription factors are preferred (Omer et al., Biochem. Biophys. Res.

Comm. 256: 584-590 (1999)), in particular amino acids 2-100 of human LEF-1 or amino acids 1-80 of human TCF-4.

Many reporter genes and transcriptional regulatory elements are known to those of skill in the art and others may be identified or synthesized by methods known to those of skill in the art. Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) Eur. J. Biochem. 182: 231-238, Hall et al. (1983) J. Mol. Appl. Gen. 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol. 216:362-368); β -lactamase or GST.

beta-Catenin accumulation may be either constitutively induced or regulatably induced. Constitutive induction of beta-catenin may be achieved by a variety of methods including, for example, engineering the host cell to express stabilizing mutations of beta-catenin, loss of function or dominant negative mutations of GSK-3 beta (Hedgepeth et al., Mol. Cell Biol. 19:7147-57 (1999) and Hedgepeth et al., Dev. Biol. 185: 82-91 (1997)), mutations in Axin, truncation mutations of APC, etc. Regulatable induction of beta-catenin may be achieved by treating the transfected cell with a component capable of stabilizing beta-catenin. Preferably the level of beta-catenin induction could be controlled based on the amount of the regulatable component added. Additionally, it would be preferred if the beta-catenin induction was reversible, so that upon removal of the component, induction of beta-catenin accumulation would cease. Preferred components for induction of beta-catenin are lithium salts, for example, lithium chloride.

The level of transcription of the gene under transcriptional control of beta-catenin may be measured by a variety of methods depending upon the gene being expressed. For example, the level of transcript may be measured directly by a variety of methods well known to the skilled in the art, including, for example, northern blotting, RT-PCR, probe hybridization, differential display, etc. Alternatively, the level of the protein encoded by the gene may be measured by a

variety of methods well known to the skilled in the art, including, for example, western blotting, immunoprecipitation, detection of enzymatic activity, etc. Additionally, transcription of a selectable marker may be identified by detecting the trait attributable to the marker, such as, for example, antibiotic resistance, drug
5 resistance, sensitivity to enzymes which affect colorimetric changes such as β -galactosidase, etc. Preferably, the level of transcript may be measured spectroscopically through detection of a reporter gene such as Luciferase, green fluorescent protein, etc.

In a preferred embodiment of the invention, a method for monitoring beta-catenin mediated transcription is featured. The method involves transfecting a cell
10 with a first nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain of a transcription factor fused to a beta-catenin binding domain, transfecting the cell with a second nucleic acid construct comprising coding sequences for a gene located downstream from sequences recognized by the DNA
15 binding domain of the chimeric protein encoded for by the first nucleic acid construct, inducing beta-catenin accumulation, and measuring the level of transcription of the gene encoded for by the second nucleic acid construct.

In a specific embodiment, an epithelial cell line is transfected with a reporter plasmid and an effector plasmid. The reporter plasmid contains binding sites for
20 yeast GAL4 positioned upstream from the firefly luciferase open reading frame. The effector plasmid contains sequences encoding for a beta-catenin binding domain from either a LEF or TCF transcription factor fused in frame, downstream, of sequences encoding for a GAL4 DNA binding domain. The cells are then induced to accumulate beta-catenin and the level of beta-catenin mediated transcription of the
25 luciferase gene is measured.

In exemplary embodiments, the reporter plasmid comprises at least one copy of a binding site for yeast GAL-4 positioned adjacent to a minimal TATA element. These control sequences are located upstream from the firefly luciferase open reading frame. In one embodiment, the effector plasmid encodes for a chimeric
30 protein fusion comprising the DNA binding domain of yeast GAL-4 (amino acids 1-147 of SEQ ID No. 6) fused to the beta-catenin binding domain of human LEF-1 (amino acids 2-100 of SEQ ID No. 4). In another embodiment, the effector plasmid

encodes for a chimeric protein fusion comprising the DNA binding domain of yeast GAL-4 (amino acids 1-147 of SEQ ID No. 6) fused to the beta-catenin binding domain of mouse TCF-4 (amino acids 1-80 of SEQ ID No. 2).

Another embodiment of the invention features a method for selective
5 inducing death of a cell containing a Wnt signaling pathway. The method involves transfecting a cell with a first nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain of a transcription factor fused to a beta-catenin binding domain and transfecting the cell with a second nucleic acid construct comprising coding sequences for a toxin gene located downstream from sequences
10 recognized by the DNA binding domain of the chimeric protein encoded for by the first nucleic acid construct, wherein the toxin gene is expressed in the presence of beta-catenin and is capable of inducing cell death. Examples of toxin genes include diphtheria toxin, ricin, cytokine genes, tumor suppressor genes (e.g., p53), DNA sequences that yield anti-sense RNA to oncogenes, genes that induce apoptosis, etc.
15 Such first and second nucleic acid constructs may introduced into an animal as a gene therapy treatment for selectively killing cells containing a Wnt signaling pathway.

Another embodiment of the invention features a method for constructing a cell line for screening compounds capable of modulating beta-catenin mediated
20 signal transduction. The method involves transfecting a cell with a variety of potential constructs which may be responsive to beta-catenin mediated signal transduction, inducing beta-catenin accumulation and comparing levels of beta-catenin mediated signal transduction obtained with the different constructs. Preferably the constructs contain an easily detectable reporter gene under
25 transcriptional control of beta-catenin.

A further embodiment of the invention features a method for identifying a compound capable of affecting Wnt mediated signal transduction. The method involves transfecting a cell with a first nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain of a transcription factor fused
30 to a beta-catenin binding domain, transfecting the cell with a second nucleic acid construct comprising coding sequences for a gene located downstream from sequences recognized by the DNA binding domain of the chimeric protein encoded

for by the first nucleic acid construct, inducing beta-catenin accumulation, contacting the cell with a test compound, and measuring the level of transcription of the gene encoded for by the second nucleic acid construct. The cell may be contacted with the test compound before, after or concurrently with induction of
5 beta-catenin production. Preferably, the level of transcription of the gene under the transcriptional control of beta-catenin is compared in the presence and absence of the test compound so as to be able to determine the effects of the test compound on beta-catenin mediated signal transduction.

The test compound may be any type of molecule which may affect beta-
10 catenin mediated signal transduction, including, for example, polypeptides, nucleic acids, carbohydrates, small organic molecules, etc. Preferably, the test compound is a member of a library of natural or synthetic compounds.

In a particularly preferred embodiment, the methods of the invention are amenable to automated, cost-effective high throughput screening.

15 In certain embodiments of the subject method, it will be desirable to monitor the growth state of cells in the culture, e.g., cell proliferation, differentiation and/or cell death. Methods of measuring cell proliferation are well known in the art and most commonly include determining DNA synthesis characteristic of cell replication. There are numerous methods in the art for measuring DNA synthesis,
20 any of which may be used according to the invention. In an embodiment of the invention, DNA synthesis has been determined using a radioactive label (³H-thymidine) or labeled nucleotide analogues (BrdU) for detection by immunofluorescence.

A further embodiment of the invention features a method for screening for
25 compounds that can alleviate at least one symptom of a disease associated with abnormal cellular proliferation. The method involves transfecting a cell with a first nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain of a transcription factor fused to a beta-catenin binding domain, transfecting the cell with a second nucleic acid construct comprising coding sequences for a gene
30 located downstream from sequences recognized by the DNA binding domain of the chimeric protein encoded for by the first nucleic acid construct, inducing beta-catenin accumulation, contacting the cell with a test compound, and measuring the

level of transcription of the gene encoded for by the second nucleic acid construct. The cell may be contacted with the test compound before, after or concurrently with induction of beta-catenin production. Preferably, the level of transcription of the gene under the transcriptional control of beta-catenin is compared in the presence
5 and absence of the test compound so as to be able to determine the effects of the test compound on Wnt signalling mediated cellular proliferation.

Compounds identified as modulating Wnt signaling mediated cellular proliferation may be used as therapeutics for inhibition or promotion of cell growth, upon a patient cell sample from tissue having or suspected of having a hyper- or
10 hypoproliferative disorder, respectively. Such hyperproliferative disorders include certain cancers such as colorectal, desmoid, endometrial, gastric, hepatocellular, hepatoblastoma, kidney (e.g. Wilm's tumor), medulloblastoma, melanoma, ovarian, pancreatic tumors, pilomatricoma, prostate, thyroid, uterine, etc. but are not limited to those described herein. Hypoproliferative disorders include diseases or conditions
15 associated with insufficient cell proliferation, such as stimulation of tissue repair, tissue regeneration, wound healing, neovascularization, etc. but are not limited to those described herein.

A further embodiment of the invention features a method for identifying a compound capable of affecting Wnt mediated embryonic development. The method
20 involves transfecting a cell with a first nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain of a transcription factor fused to a beta-catenin binding domain, transfecting the cell with a second nucleic acid construct comprising coding sequences for a gene located downstream from sequences recognized by the DNA binding domain of the chimeric protein encoded
25 for by the first nucleic acid construct, inducing beta-catenin accumulation, contacting the cell with a test compound, and measuring the level of transcription of the gene encoded for by the second nucleic acid construct. The cell may be contacted with the test compound before, after or concurrently with induction of beta-catenin production. Preferably, the level of transcription of the gene under the
30 transcriptional control of beta-catenin is compared in the presence and absence of the test compound so as to be able to determine the effects of the test compound on Wnt signaling mediated embryonic development.

Compounds identified as modulating Wnt signaling mediated embryonic development may be used as therapeutics for modulation of embryonic development or cellular differentiation. For example, the compounds may be used as activators or repressors of a variety of developmental processes including for example, establishment of embryonic axes, pattern formation of early mesoderm and ectoderm (Montross et al., J. Cell Sci. 113: 1759-70 (2000)), thymocyte differentiation (Verbeek et al., Nature 374: 70-74 (1995)), axonal growth of neurons during synapse formation in the developing brain (Takahashi et al., J. Neurochem. 73:2073-2083 (1999)), development of the brain, mesenchyme below the epidermis, lung mesenchyme, and kidney (Behrens, Ann. NY Acad. Sci. 910: 21-33 (2000)), etc.

The invention also provides nucleic acid constructs, chimeric proteins and cell lines for carrying out the methods of the invention.

In one embodiment, the invention features a nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain of a transcription factor fused to a beta-catenin binding domain. In exemplary embodiments, the nucleic acid construct encodes for the DNA binding domain of yeast GAL-4 (amino acids 1-147 of SEQ ID No. 6) fused to the beta-catenin binding domain of either human LEF-1 (amino acids 2-100 of SEQ ID No. 4) or mouse TCF-4 (amino acids 1-80 of SEQ ID No. 2).

In another embodiment, the invention features a chimeric protein comprising a DNA binding domain of a transcription factor fused to a beta-catenin binding domain. In exemplary embodiments, the chimeric protein comprises the DNA binding domain of yeast GAL-4 (amino acids 1-147 of SEQ ID No. 6) fused to the beta-catenin binding domain of either human LEF-1 (amino acids 2-100 of SEQ ID No. 4) or mouse TCF-4 (amino acids 1-80 of SEQ ID No. 2).

A further embodiment of the invention features a cultured cell expressing a chimeric protein comprising a DNA binding domain of a transcription factor fused to a beta-catenin binding domain. In exemplary embodiments, the chimeric protein comprises the DNA binding domain of yeast GAL-4 (amino acids 1-147 of SEQ ID No. 6) fused to the beta-catenin binding domain of either human LEF-1 (amino acids 2-100 of SEQ ID No. 4) or mouse TCF-4 (amino acids 1-80 of SEQ ID No. 2).

Yet another embodiment of the invention features a cultured cell comprising a nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain of a transcription factor fused to a beta-catenin binding domain. In exemplary embodiments, the nucleic acid construct encodes for the DNA binding domain of yeast GAL-4 (amino acids 1-147 of SEQ ID No. 6) fused to the beta-catenin binding domain of either human LEF-1 (amino acids 2-100 of SEQ ID No. 4) or mouse TCF-4 (amino acids 1-80 of SEQ ID No. 2).

In another embodiment, the invention features a cultured cell comprising a first nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain of a transcription factor fused to a beta-catenin binding domain, and a second nucleic acid construct comprising coding sequences for a gene located downstream from sequences recognized by the DNA binding domain of the chimeric protein encoded for by the first nucleic acid construct. In exemplary embodiments, the first nucleic acid construct comprises at least one copy of a 17 base pair binding site for yeast GAL-4 positioned adjacent to a minimal TATA element. These control sequences are located upstream from the firefly luciferase open reading frame. The second nucleic acid construct comprises nucleic acid sequences encoding for the DNA binding domain of yeast GAL-4 (amino acids 1-147 of SEQ ID No. 6) fused to the beta-catenin binding domain of either human LEF-1 (amino acids 2-100 of SEQ ID No. 4) or mouse TCF-4 (amino acids 1-80 of SEQ ID No. 2).

Preferably, the nucleic acid constructs of the invention would be contained on a plasmid or vector. In general, it will be desirable that the vector be capable of replication in the host cell. It may be a DNA which is integrated into the host genome, and thereafter is replicated as a part of the chromosomal DNA, or it may be DNA which replicates autonomously, as in the case of a plasmid. In the latter case, the vector will include an origin of replication which is functional in the host. In the case of an integrating vector, the vector may include sequences which facilitate integration, e.g., sequences homologous to host sequences, or encoding integrases.

Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are known in the art, and are described in, for example, Powels et al. (*Cloning Vectors: A Laboratory Manual*, Elsevier, New

York, 1985). Mammalian expression vectors may comprise non-transcribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a poly-adenylation site, splice donor and acceptor sites, and transcriptional termination sequences. The expression vector may be either linear or circular.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

The transcriptional and translational control sequences in expression vectors to be used in transforming mammalian cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from Polyoma, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a heterologous DNA sequence. The early and late promoters are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al.(1978) *Nature* 273:111)

In other embodiments, the use of viral transfection can provide stably integrated copies of the reporter or effector constructs. In particular, the use of retroviral, adenoviral or adeno-associated viral vectors is contemplated as a means for providing a stably transfected cell line.

5 In some instances, it may be desirable to utilize an insect cell host. In such embodiments, recombinant polypeptides can be expressed by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the β -gal
10 containing pBlueBac III).

In constructing suitable expression plasmids, the termination sequences associated with these genes, or with other genes which are efficiently expressed in the host cell, may also be ligated into the expression vector 3' of the heterologous coding sequences to provide polyadenylation and termination of the mRNA.

15 The methods of the invention may also be carried out in a cell free system using purified components.

After identifying certain test compounds in the subject assay, e.g., as potential agonists or antagonists of beta-catenin mediated signal transduction, the practitioner of the subject assay will continue to test the efficacy and specificity of the
20 selected compounds both *in vitro* and *in vivo*. Whether for subsequent *in vivo* testing, or for administration to an animal as an approved drug, agents identified in the subject assay can be formulated in pharmaceutical preparations for *in vivo* administration to an animal, preferably a human.

Wnt-Therapeutics and Uses Thereof

25 Broadly, in one embodiment, this invention provides Agonist and Antagonist therapeutics, which can either mimic or potentiate Wnt function, i.e., the activity of the Wnt-signaling pathway or which can antagonize Wnt function, i.e., the activity of the Wnt-signaling pathway. The Antagonist Therapeutics of the invention are those Therapeutics which antagonize, or inhibit, a Wnt function. Such Antagonist
30 Therapeutics are most preferably identified by the assays described herein or by use of known convenient *in vitro* assays, e.g., based on their ability to modulate and/or inhibit the interaction between beta-catenin and a LEF/TCF family member. In a

preferred embodiment, the Antagonist Therapeutic is a protein or derivative thereof comprising a functionally active fragment that can inhibit the interaction between beta-catenin to a LEF/TCF family member, such as for example, Duplin (Sakamoto et al., J. Biol. Chem., 275:42 (2000): 32871-8), dominant negative forms of beta-catenin (Zhurinsky et al., Mol. Cell Biol. 20: 4238-52 (2000)), or an antibody thereto, or an analog/competitive inhibitor of a Wnt signal-transducing region, etc. It should be noted that in certain instances, an Antagonist Therapeutics may alternatively act as an Agonist Therapeutic, depending on the developmental history of the tissue being exposed to the Therapeutic; preferably, suitable in vitro or in vivo assays, as described herein, may be utilized to determine the effect of a specific Therapeutic and whether its administration is indicated for treatment of the affected tissue.

The Agonist Therapeutics of the invention, as described herein, promote, mimic, or potentiate the interaction between beta-catenin and a member of the TCF/LEF family. Such Agonist Therapeutics include but are not limited to proteins and derivatives comprising the portions that mediate binding to TCF/LEF, and nucleic acids encoding the foregoing (which can be administered to express their encoded products in vivo).

The Agonist and Antagonist Therapeutics of the invention have therapeutic utility for disorders of cell fate. The Agonist Therapeutics are administered therapeutically (including prophylactically): (1) in diseases or disorders involving an absence or decreased (relative to normal, or desired) levels of Wnt function, for example, in patients where any member of the Wnt-signaling pathway is lacking, genetically defective, biologically inactive or underactive, or underexpressed; and (2) in diseases or disorders wherein in vitro (or in vivo) assays indicate the utility of Wnt agonist administration. In preferred embodiments, the Agonist Therapeutic is administered (1) in diseases or disorders involving an absence or decreased levels of Wnt function, for example, in patients where beta-catenin is lacking, genetically defective, biologically inactive or underactive, or underexpressed; and (2) in diseases or disorders wherein in vitro (or in vivo) assays indicate the utility of Wnt agonist administration. The absence or decreased levels in Wnt pathway function can be readily detected, e.g., by obtaining a patient tissue sample (e.g., from biopsy

tissue) and assaying it in vitro for protein levels, structure and/or activity of the expressed beta-catenin protein. Many methods standard in the art can be thus employed, including but not limited to immunoassays to detect and/or visualize beta-catenin protein (e.g., Western blot, immunoprecipitation followed by sodium
5 dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.; and/or hybridization assays to detect expression of a member of the Wnt-signaling pathway, by detecting and/or visualizing for example beta-catenin mRNA (e.g., Northern assays, dot blots, in situ hybridization, etc.).

In vitro assays which can be used to determine whether administration of a
10 specific Agonist Therapeutic or Antagonist Therapeutic is indicated, include in vitro cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a Therapeutic, and the effect of such Therapeutic upon the tissue sample is observed. In one embodiment, where the patient has a malignancy, a sample of cells from such malignancy is plated out or grown in
15 culture, and the cells are then exposed to a Therapeutic. A Therapeutic which inhibits survival or growth of the malignant cells (e.g., by promoting terminal differentiation) is selected for therapeutic use in vivo. Many assays standard in the art can be used to assess such survival and/or growth; for example, cell proliferation can be assayed by measuring ³H-thymidine incorporation, by direct cell count, by
20 detecting changes in transcriptional activity of known genes such as proto-oncogenes (e.g., fos, myc) or cell cycle markers; cell viability can be assessed by trypan blue staining, differentiation can be assessed visually based on changes in morphology, etc. In a specific aspect, the malignant cell cultures are separately exposed to (1) an Agonist Therapeutic, and (2) an Antagonist Therapeutic; the result
25 of the assay can indicate which type of Therapeutic has therapeutic efficacy.

In another embodiment, a Therapeutic is indicated for use which exhibits the desired effect, inhibition or promotion of cell growth, upon a patient cell sample from tissue having or suspected of having a hyper- or hypoproliferative disorder, respectively. Such hyperproliferative disorders include certain cancers such as
30 colorectal, desmoid, endometrial, gastric, hepatocellular, hepatoblastoma, kidney (e.g. Wilm's tumor), medulloblastoma, melanoma, ovarian, pancreatic tumors, pilomatricoma, prostate, thyroid, uterine, etc. but are not limited to those described

herein. Hypoproliferative disorders include diseases or conditions associated with insufficient cell proliferation, such as stimulation of tissue repair, tissue regeneration, wound healing, neovascularization, etc. but are not limited to those described herein.

5 In various specific embodiments, in vitro assays can be carried out with representative cells of cell types involved in a patient's disorder, to determine if a Therapeutic has a desired effect upon such cell types.

 In another embodiment, cells of a patient tissue sample suspected of being pre-neoplastic are similarly plated out or grown in vitro, and exposed to a
10 Therapeutic. The Therapeutic which results in a cell phenotype that is more normal (i.e., less representative of a pre-neoplastic state, neoplastic state, malignant state, or transformed phenotype) is selected for therapeutic use. Many assays standard in the art can be used to assess whether a pre-neoplastic state, neoplastic state, or a transformed or malignant phenotype, is present. For example, characteristics
15 associated with a transformed phenotype (a set of in vitro characteristics associated with a tumorigenic ability in vivo) include a more rounded cell morphology, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, release of proteases such as plasminogen activator, increased sugar transport, decreased serum requirement, expression of fetal antigens, disappearance of the
20 250,000 dalton surface protein, etc. (see Luria et al., 1978, General Virology, 3d Ed., John Wiley & Sons, New York pp. 436-446).

 In other specific embodiments, the in vitro assays described supra can be carried out using a cell line, rather than a cell sample derived from the specific patient to be treated, in which the cell line is derived from or displays
25 characteristic(s) associated with the malignant, neoplastic or pre-neoplastic disorder desired to be treated or prevented, or is derived from the neural or other cell type upon which an effect is desired, according to the present invention.

 The Antagonist Therapeutics are administered therapeutically (including prophylactically): (1) in diseases or disorders involving increased (relative to
30 normal, or desired) levels of Wnt function, for example, where any member of the Wnt signaling pathway, such as for example, the beta-catenin protein is overexpressed or overactive; and (2) in diseases or disorders wherein in vitro (or in

vivo) assays indicate the utility of Wnt antagonist administration. The increased levels of beta-catenin function can be readily detected by methods such as those described above, by quantifying protein and/or RNA. In vitro assays with cells of patient tissue sample or the appropriate cell line or cell type, to determine
5 therapeutic utility, can be carried out as described above.

In yet another embodiment, the Antagonist Therapeutic of this invention includes within its scope, antibodies and fragments containing the binding domain thereof, directed against beta-catenins. Accordingly, beta-catenin proteins, fragments or analogs or derivatives thereof, in particular, human beta-catenin
10 proteins or fragments thereof, may be used as immunogens to generate anti-beta-catenin protein antibodies. Such antibodies can be polyclonal, monoclonal, chimeric, single chain, Fab fragments, or from an Fab expression library. In another embodiment, antibodies specific to human beta-catenin are produced.

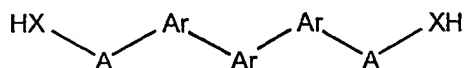
Various procedures known in the art may be used for the production of
15 polyclonal antibodies to a beta-catenin protein or peptide. For the production of antibody, various host animals can be immunized by injection with the native beta-catenin protein, or a synthetic version, or fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not
20 limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *corynebacterium parvum*.

25 For preparation of monoclonal antibodies directed toward a beta-catenin protein sequence, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256, 495-497), as well as the trioma technique, the human B-cell hybridoma
30 technique (Kozbor et al., 1983, Immunology Today 4, 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Antibody fragments which contain the idiotype (binding domain) of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by
 5 reducing the disulfide bridges of the F(ab')₂ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Small Molecule Antagonists

In certain embodiments, an antagonist useful in the compositions and
 10 methods of the invention has a structure of general formula I:



wherein, as valence and stability permit,

X, independently for each occurrence, represents O, S, or NR;

R, independently for each occurrence, represents lower alkyl, lower
 15 alkylene, or aralkyl, preferably lower alkyl;

A, independently for each occurrence, represents substituted or unsubstituted lower alkylene; and

Ar, independently for each occurrence, represents a substituted or unsubstituted aryl or heteroaryl ring.

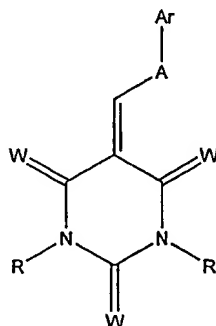
20 In certain embodiments, the two bonds from Ar (e.g., to Ar and Ar or A) are disposed in a *meta*- or 1,3-relationship on Ar. Thus, for example, if an occurrence of Ar represents a 5-membered heteroaryl ring, the two bonds may be located at the 2 and 5 positions of Ar, e.g., as in Compound B, wherein each occurrence of Ar is a thiophene ring.

25 In certain embodiments, each occurrence of Ar is selected from substituted or unsubstituted phenyl, thienyl, oxazolyl, pyrrolyl, furanyl, thiazolyl, and pyridyl rings. In certain embodiments, all three occurrences of Ar are the same substituted or unsubstituted aryl or heteroaryl ring, e.g., selected from substituted or unsubstituted phenyl, thienyl, oxazolyl, pyrrolyl, furanyl, thiazolyl, and pyridyl
 30 rings. In certain embodiments, at least two or even all three occurrences of Ar independently represent a substituted or unsubstituted thiophene ring.

In certain embodiments, A, independently for each occurrence, is selected from C1-C4 alkylene, such as -CH(Me)-, methylene, ethylene, -CH₂C(Me)₂-, etc.

In certain embodiments, each occurrence of X represents O.

In certain other embodiments, an antagonist useful in the compositions and
5 methods of the invention has a structure of general formula II:



wherein, as valence and stability permit,

W, independently for each occurrence, represents O or S, preferably O;

R, independently for each occurrence, represents lower alkyl, lower
10 alkylene, or aralkyl, preferably lower alkyl;

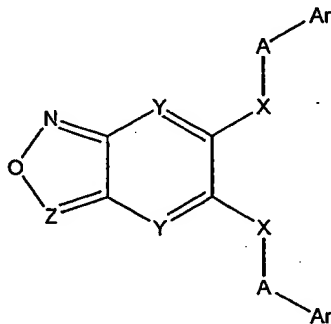
A is absent or represents substituted or unsubstituted lower alkylene; and

Ar represents a substituted or unsubstituted aryl or heteroaryl ring.

In certain embodiments, Ar represents a substituted or unsubstituted phenyl
ring.

15 In certain embodiments, A, independently for each occurrence, is selected from C1-C4 alkylene, such as -CH(Me)-, methylene, ethylene, -CH₂C(Me)₂-, etc.

In certain other embodiments, an antagonist useful in the compositions and methods of the invention has a structure of general formula III:



20 wherein, as valence and stability permit,

X, independently for each occurrence, is absent or represents O, S, or NR;

R, independently for each occurrence, represents lower alkyl, lower alkylene, or aralkyl, preferably lower alkyl;

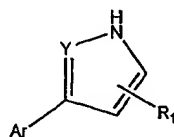
Y and Z, independently for each occurrence, represent N or CH;

A, independently for each occurrence, is absent or represents substituted or unsubstituted lower alkylene, and

Ar, independently for each occurrence, represents a substituted or unsubstituted aryl or heteroaryl ring.

In certain embodiments, at least one occurrence of Y represents N, preferably both occurrences. In certain embodiments, Z represents N. In certain embodiments, X represents NR, such as NH. In certain embodiments, at least one occurrence of Ar represents a substituted or unsubstituted phenyl ring, preferably both occurrences.

In certain other embodiments, an antagonist useful in the compositions and methods of the invention has a structure of general formula IV:



wherein, as valence and stability permit,

Y represents N or CH;

R₁ represents from 1-3 substituents to the ring to which it is attached, independently selected from hydrogen, halogen, alkyls, alkenyl, alkynyl, aryl, hydroxyl, alkoxyl, silyloxy, amino, nitro, thiol, amines, imines, amides, phosphoryls, phosphonates, phosphines, carbonyls, carboxyls, carboxamides, anhydrides, silyls, ethers, thioethers, alkylsulfonyls, arylsulfonyls, selenoethers, ketones, aldehydes, esters, or -(CH₂)_n-R₈;

R₈, independently for each occurrence, represents H, lower alkyl, aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., -(CH₂)_naryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., -(CH₂)_nheteroaralkyl-);

Ar, independently for each occurrence, represents a substituted or unsubstituted aryl or heteroaryl ring, preferably a substituted or unsubstituted phenyl ring; and

n, individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, R1 represents a substituted alkyl, such as a perfluoroalkyl. In certain embodiments, Ar represents a substituted or unsubstituted
5 phenyl ring.

In certain embodiments, a compound which is an agonist or antagonist useful in the present invention is chosen to be selective for the Wnt pathway over protein kinases, such as PKC, e.g., the compound modulates the activity of Wnt at least an order of magnitude more strongly than it modulates the activity of a protein kinase,
10 preferably at least two orders of magnitude more strongly, even more preferably at least three orders of magnitude more strongly. Thus, for example, a preferred agonist or antagonist may modulate Wnt activity with an EC_{50} or IC_{50} at least an order of magnitude lower than its EC_{50}/IC_{50} for inhibition of PKC, preferably at least two orders of magnitude lower, even more preferably at least three orders of
15 magnitude lower. In certain embodiments, a Wnt antagonist inhibits PKC with a K_i greater than 10 nM, greater than 100 nM, preferably greater than 1 μ M, even more preferably greater than 10 μ M or 100 μ M.

Combinatorial Libraries

The compounds of the present invention, particularly libraries of variants
20 having various representative classes of substituents, are amenable to combinatorial chemistry and other parallel synthesis schemes (see, for example, PCT WO 94/08051). The result is that large libraries of related compounds, e.g., a variegated library of compounds represented above, can be screened rapidly in high throughput assays in order to identify potential Wnt modulators lead compounds, as well as to
25 refine the specificity, toxicity, and/or cytotoxic-kinetic profile of a lead compound. For instance, assays as described herein can be used to screen a library of the subject compounds for those having antagonist activity towards the Wnt pathway.

Simply for illustration, a combinatorial library for the purposes of the present invention is a mixture of chemically related compounds which may be screened
30 together for a desired property. The preparation of many related compounds in a single reaction greatly reduces and simplifies the number of screening processes

which need to be carried out. Screening for the appropriate physical properties can be done by conventional methods.

Diversity in the library can be created at a variety of different levels. For instance, the substrate aryl groups used in the combinatorial reactions can be diverse
5 in terms of the core aryl moiety, e.g., a variegation in terms of the ring structure, and/or can be varied with respect to the other substituents.

A variety of techniques are available in the art for generating combinatorial libraries of small organic molecules such as the subject Wnt modulators. See, for example, Blondelle et al. (1995) Trends Anal. Chem. 14:83; the Affymax U.S.
10 Patents 5,359,115 and 5,362,899; the Ellman U.S. Patent 5,288,514; the Still et al. PCT publication WO 94/08051; the ArQule U.S. Patents 5,736,412 and 5,712,171; Chen et al. (1994) JACS 116:2661; Kerr et al. (1993) JACS 115:252; PCT publications WO92/10092, WO93/09668 and WO91/07087; and the Lerner et al. PCT publication WO93/20242). Accordingly, a variety of libraries on the order of
15 about 100 to 1,000,000 or more diversomers of the subject Wnt modulators can be synthesized and screened for particular activity or property.

In an exemplary embodiment, a library of candidate Wnt modulators diversomers can be synthesized utilizing a scheme adapted to the techniques described in the Still et al. PCT publication WO 94/08051, e.g., being linked to a
20 polymer bead by a hydrolyzable or photolyzable group, optionally located at one of the positions of the candidate antagonists or a substituent of a synthetic intermediate. According to the Still et al. technique, the library is synthesized on a set of beads, each bead including a set of tags identifying the particular diversomer on that bead. The bead library can then be "plated" with cells as described herein
25 capable of indicating modulation of Wnt activity by a test compound. The diversomers can be released from the bead, e.g. by hydrolysis.

Compounds prepared by any of the above techniques can then be tested in one or more assays as described herein, e.g., in a high-throughput assay, to measure their activity towards modulation of Wnt pathway signaling. Repeated iterations of
30 synthesis and testing can be used as part of a medicinal chemistry program to identify compounds which have increased activity, reduced side-effects, etc.

Pharmaceutical Compositions

The compounds selected in the subject assay, or a pharmaceutically acceptable salt thereof, may accordingly be formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the compound, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations". Based on the above, such pharmaceutical formulations include, although not exclusively, solutions or freeze-dried powders of the compound in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered media at a suitable pH and is osmotic with physiological fluids. In preferred embodiment, the compound can be disposed in a sterile preparation for topical and/or systemic administration. In the case of freeze-dried preparations, supporting excipients such as, but not exclusively, mannitol or glycine may be used and appropriate buffered solutions of the desired volume will be provided so as to obtain adequate isotonic buffered solutions of the desired pH. Similar solutions may also be used for the pharmaceutical compositions of compounds in isotonic solutions of the desired volume and include, but not exclusively, the use of buffered saline solutions with phosphate or citrate at suitable concentrations so as to obtain at all times isotonic pharmaceutical preparations of the desired pH, (for example, neutral pH).

Gene Therapy

The invention provides methods for selectively inducing death of cells containing a Wnt signaling pathway. According to the methods of the invention, the nucleic acid constructs are administered to a subject having a disease associated
5 with aberrant Wnt signaling, such as a hyperproliferative disorder, including, for example a variety of cancers and leukemias, in particular, cancers such as colorectal, desmoid, endometrial, gastric, hepatocellular, hepatoblastoma, kidney (e.g. Wilm's tumor), medulloblastoma, melanoma, ovarian, pancreatic tumors, pilomatricoma, prostate, thyroid and uterine.

10 In one aspect of the invention, the nucleic acid constructs of the invention may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively transfecting cells *in vivo* with a nucleic acid construct of the invention. Approaches include insertion of the subject gene in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and
15 herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors can be used to transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO_4
20 precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically.

A preferred approach for *in vivo* introduction of the nucleic acid constructs
25 of the invention into a cell is by use of a viral vector containing the subject nucleic acid sequences. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector are expressed efficiently in cells which have taken up viral vector nucleic acid.

30 Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient

delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag*, *pol*, *env*) has been replaced by nucleic acid construct of the invention, rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in *Current Protocols in Molecular Biology*, Ausubel, F.M. et al., (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψ Crip, ψ Cre, ψ 2 and ψ Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including neural cells, epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis et al., (1985) *Science* 230:1395-1398; Danos and Mulligan, (1988) *PNAS USA* 85:6460-6464; Wilson et al., (1988) *PNAS USA* 85:3014-3018; Armentano et al., (1990) *PNAS USA* 87:6141-6145; Huber et al., (1991) *PNAS USA* 88:8039-8043; Ferry et al., (1991) *PNAS USA* 88:8377-8381; Chowdhury et al., (1991) *Science* 254:1802-1805; van Beusechem et al., (1992) *PNAS USA* 89:7640-7644; Kay et al., (1992) *Human Gene Therapy* 3:641-647; Dai et al., (1992) *PNAS USA* 89:10892-10895; Hwu et al., (1993) *J. Immunol.* 150:4104-4115; U.S. Patent NO: 4,868,116; U.S. Patent NO: 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al., (1989) *PNAS USA* 86:9079-9083; Julan et al., (1992) *J. Gen Virol* 73:3251-3255; and Goud et al., (1983) *Virology* 163:251-254); or coupling cell surface ligands to the viral *env* proteins (Neda et al., (1991) *J. Biol. Chem.* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, and can also be used to convert an ecotropic vector in to an amphotropic vector.

Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes a gene product of interest, but is inactivate in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al., (1988) *BioTechniques* 6:616; Rosenfeld et al., (1991) *Science* 252:431-434; and Rosenfeld et al., (1992) *Cell* 68:143-155). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al., (1992) cited *supra*), endothelial cells (Lemarchand et al., (1992) *PNAS USA* 89:6482-6486), hepatocytes (Herz and Gerard, (1993) *PNAS USA* 90:2812-2816) and muscle cells (Quantin et al., (1992) *PNAS USA* 89:2581-2584). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but

remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al., (1979) *Cell* 16:683; Berkner et al., *supra*; and Graham et al., in *Methods in Molecular Biology*, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted chimeric sequences (e.g., encoding for a chimeric protein comprising a DNA binding domain of a transcription factor fused to a beta-catenin binding domain) can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the viral E3 promoter, or exogenously added promoter sequences.

Yet another viral vector system useful for delivery of the subject nucleic acid constructs is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review, see Muzyczka et al., *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al., (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al., (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al., (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al., (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al., (1984) *PNAS USA* 81:6466-6470; Tratschin et al., (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al., (1988) *Mol. Endocrinol.* 2:32-39; Tratschin et al., (1984) *J. Virol.* 51:611-619; and Flotte et al., (1993) *J. Biol. Chem.* 268:3781-3790).

Other viral vector systems that may have application in gene therapy have been derived from herpes virus, vaccinia virus, and several RNA viruses. In particular, herpes virus vectors may provide a unique strategy for persistence of the nucleic acid constructs of the invention in cells of the central nervous system and ocular tissue (Pepose et al., (1994) *Invest Ophthalmol Vis Sci* 35:2662-2666)

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to deliver the nucleic acid constructs of the invention to the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the subject nucleic acid construct by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, the nucleic acid constructs of the invention can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al., (1992) *No Shinkei Geka* 20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075). For example, lipofection of neuroglioma cells can be carried out using liposomes tagged with monoclonal antibodies against glioma-associated antigen (Mizuno et al., (1992) *Neurol. Med. Chir.* 32:873-876).

In yet another illustrative embodiment, the gene delivery system comprises an antibody or cell surface ligand which is cross-linked with a gene binding agent such as poly-lysine (see, for example, PCT publications WO93/04701, WO92/22635, WO92/20316, WO92/19749, and WO92/06180). For example, the subject nucleic acid constructs can be used to transfect specific cells *in vivo* using a soluble polynucleotide carrier comprising an antibody conjugated to a polycation, e.g. poly-lysine (see U.S. Patent 5,166,320). It will also be appreciated that effective delivery of the subject nucleic acid constructs via -mediated endocytosis can be improved using agents which enhance escape of the gene from the endosomal structures. For instance, whole adenovirus or fusogenic peptides of the influenza HA

gene product can be used as part of the delivery system to induce efficient disruption of DNA-containing endosomes (Mulligan et al., (1993) *Science* 260:926; Wagner et al., (1992) *PNAS USA* 89:7934; and Christiano et al., (1993) *PNAS USA* 90:2122).

In clinical settings, the gene delivery systems can be introduced into
5 a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the construct in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the
10 transcriptional regulatory sequences controlling expression of the gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al., (1994) *PNAS USA* 91: 3054-3057).

15 (iv) Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

20 **Example 1: Regulation of beta-Catenin Activity Using Lithium Chloride**

HEK-293 cells (human embryonal kidney cells, ATCC CRL-1573) were transiently transfected with a reporter plasmid alone (E1b-luc only), the reporter plasmid plus an expression vector containing a GAL-4 DBD (DNA binding domain) without a catenin binding domain (GAL-DBD), the reporter plasmid plus an
25 expression vector containing a GAL-4 DBD-catenin binding domain (GAL4-LEF) or the reporter plasmid plus an expression vector containing a GAL-4 DBD fused to a fragment of the catenin binding domain lacking most of the catenin binding domain (GAL4-DNLEF).

The reporter plasmid contained five copies of a 17 bp binding site for yeast
30 GAL4 positioned adjacent to a minimal TATA element derived from the Adenovirus E1b gene. The firefly luciferase open reading frame was inserted downstream of these control elements.

The effector plasmid contained DNA sequences encoding for the beta-catenin binding domain of human LEF-1 (amino acids 2-100) inserted downstream of sequences encoding the DNA binding domain of yeast GAL4 (GAL4 DBD: amino acids 1-147). Promoter/enhancer elements from the SV40 virus were
5 positioned upstream of these protein coding sequences.

The transfected HEK-293 cells were seeded in 96-well assay plates (typically with about 50,000 cells per well) in normal tissue culture medium. The following day, the cells were re-fed with medium containing varying amounts of LiCl (0-100 mM). After a 12-24 hour incubation in the LiCl containing medium,
10 Luciferase activity was measured.

As shown in Figure 1, dose-dependent LiCl induction of Luciferase requires a functional beta-catenin binding domain be fused to the GAL4 DBD (GAL4-LEF). There was no response using a mutant LEF-1 fragment lacking most of the beta-catenin binding domain (GAL4-DNLEF).

15 **Example 2: Construction of a Cell Line for Screening Compounds Effecting Wnt Signaling**

The TCF2 cell line was produced by transfecting HEK-293 cells with a linearized reporter plasmid encoding for luciferase, an effector plasmid encoding for GAL-4 fusions and a plasmid containing a selectable marker (Zeocin, Invitrogen).

20 The reporter plasmid contained five copies of a 17 bp binding site for yeast GAL4 positioned adjacent to a minimal TATA element derived from the Adenovirus E1b gene. The firefly luciferase open reading frame was inserted downstream of these control elements.

The effector plasmid contained DNA sequences encoding for the beta-catenin binding domain of murine TCF-4 (amino acids 1-80) inserted downstream
25 of sequences encoding the DNA binding domain of yeast GAL4 (GAL4 DBD: amino acids 1-147). Promoter/enhancer elements from the SV40 virus were positioned upstream of these protein coding sequences.

Following selection of transfected cells based on Zeocin, individual cell lines
30 were chosen and assayed for LiCl inducible Luciferase expression as described in Example 1. Figure 2 shows the levels of Luciferase expression in response to increasing amounts of LiCl (0-150 mM) for cell line TCF2.

Cell line TCF2, or a similar cell line, can be used to screen compounds for the ability to effect Wnt signal transduction. For screening, Wnt signaling would be induced through addition of LiCl to the cells leading to an increase in the level of beta-catenin protein and beta-catenin induced transcription. Test compounds would be added just prior to, commensurate with or just after lithium chloride addition. The level of Luciferase (or other reporter gene) production would be compared in the presence and absence of the test compound to identify those compounds that had an effect on beta-catenin induced transcription.

Example 3: Screening of Test Compounds A-D for the Ability to Effect Wnt Signaling

Cell line TCF2 (described above) was stimulated with lithium chloride to induce beta-catenin accumulation. Concurrently with LiCl stimulation, cells were contacted with increasing doses of test compounds A-D (Cpd A-D) and the levels of luciferase activity were compared to the levels observed for stimulated cells (LiCl) and unstimulated control cells (NaCl). As shown in Figure 3, test compounds A-D showed dose-dependent inhibition of luciferase activity in the TCF2 cell line.

Example 4: Effect of Compound B on SW 480 and HepG2 Cells.

Ninety-six well plates were seeded with SW480 cells (15,000 cells per well; RPMI medium supplemented with 10% fetal bovine serum (FBS)) or HepG2 cells (10,000 cells per well; Minimal Essential Medium supplemented with non-essential amino acids, 1 mM sodium pyruvate and 10% FBS) and incubated at 37 °C in an atmosphere containing 5% carbon dioxide. After 24 hours, cells were switched to 0.5% serum containing medium and compound B was added at a final concentration of 0-10 µM, with the concentration of DMSO held constant at 0.2% (v/v). Following 48 h of treatment, one-tenth volume of Alamar Blue (Biosource International, Inc.) was added, and the plates were incubated an additional two hours. Conversion of Alamar Blue, an indicator of metabolic activity, was measured in a CytoFluor Series 4000 multi-well plate reader (excitation 530 nm, emission 590 nm). Values are expressed as percentage of fluorescent emission relative to control (0.2 % DMSO). The average of duplicate measurements is shown.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the

5 following claims.

All patents, publications, and other references cited above are hereby incorporated by reference in their entirety.

Claims:

1. A method for assaying beta-catenin mediated transcriptional activation, comprising
 - (a) transfecting a cell with a first nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain from a transcription factor fused to a beta-catenin binding domain,
 - (b) transfecting the cell with a second nucleic acid construct comprising coding sequences for a reporter gene located downstream from sequences recognized by the DNA binding domain of said chimeric protein encoded for by the first nucleic acid construct,
 - (c) inducing beta-catenin accumulation, and
 - (d) measuring the level of transcription of the gene encoded for by the second nucleic acid construct.
2. The method of claim 1, wherein said chimeric protein comprises a DNA binding domain from a yeast GAL-4 protein.
3. The method of claim 2, wherein said chimeric protein comprises amino acids 1-147 of the yeast GAL-4 protein.
4. The method of claim 1, wherein said chimeric protein comprises a beta-catenin binding domain from a member of the TCF/LEF family of transcription factors.
5. The method of claim 4, wherein said chimeric protein comprises a beta-catenin binding domain from a TCF transcription factor.
6. The method of claim 5, wherein said chimeric protein comprises amino acids 1-80 of the murine TCF-4 transcription factor.
7. The method of claim 1, wherein said chimeric protein comprises amino acids 1-147 of yeast GAL-4 protein and amino acids 1-80 of the murine TCF-4 transcription factor.
8. The method of claim 4, wherein said chimeric protein comprises a beta-catenin binding domain from a LEF transcription factor.
9. The method of claim 8, wherein said chimeric protein comprises amino acids 2-100 of the human LEF-1 transcription factor.

10. The method of claim 1, wherein said chimeric protein comprises amino acids 1-147 of yeast GAL-4 protein and amino acids 2-100 of the human LEF-1 transcription factor.
11. The method of claim 1, wherein said reporter gene is selected from
5 chloramphenicol acetyl transferase, luciferase, beta-galactosidase, alkaline phosphatase, beta-lactamase, horseradish peroxidase, green fluorescent protein and glutathione S-transferase.
12. The method of claim 1, wherein the reporter gene is luciferase.
13. The method of claim 1, wherein the first and second nucleic acid constructs
10 are contained on a plasmid.
14. The method of claim 13, wherein the first and second nucleic acid constructs are contained on the same plasmid.
15. The method of claim 13, wherein the first and second nucleic acid constructs are contained on different plasmids.
- 15 16. The method of claim 13, wherein the first, second or first and second nucleic acid constructs are linear.
17. The method of claim 13, wherein the first, second or first and second nucleic acid constructs are circular.
18. The method of claim 1, wherein beta-catenin accumulation is induced by
20 incubating the transfected cell with lithium chloride.
19. The method of claim 1, wherein the cell is selected from prokaryotic, yeast, plant, insect, worm, frog, fly, fish, mouse, rat, monkey, animal and mammalian cells.
20. The method of claim 19, wherein the cell is a Drosophila cell.
- 25 21. The method of claim 19, wherein the cell is a human cell.
22. The method of claim 1, wherein the cell is an epithelial cell.
23. The method of claim 22, wherein the cell is HEK-293 cell.
24. The method of claim 1, wherein the level of transcription of the reporter gene is measured spectroscopically.
- 30 25. The method of claim 1, which is adaptable to a high throughput format.
26. A method for identifying a compound capable of modulating Wnt mediated signal transduction, comprising

- (a) transfecting a cell with a first nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain from a transcription factor fused to a beta-catenin binding domain,
- (b) transfecting the cell with a second nucleic acid construct comprising coding sequences for a reporter gene located downstream from sequences recognized by the DNA binding domain of said chimeric protein encoded for by the first nucleic acid construct,
- (c) inducing beta-catenin accumulation,
- (d) contacting said cell with a test compound,
- (e) measuring the level of transcription of the reporter gene, and
- (f) comparing the level of expression of said reporter gene in the presence of said test compound and in the absence of said test compound,

wherein step (c) may occur before, after or concurrently with step (d), and wherein a change in the level of expression of said reporter gene in the presence of said compound is indicative of a compound that modulates Wnt signaling activity.

27. The method of claim 26, wherein the chimeric protein comprises a DNA binding domain from a yeast GAL-4 protein.

28. The method of claim 27, wherein the chimeric protein comprises amino acids 1-147 of the yeast GAL-4 protein.

29. The method of claim 26, wherein the chimeric protein comprises a beta-catenin binding domain from a member of the TCF/LEF family of transcription factors.

30. The method of claim 29, wherein the chimeric protein comprises a beta-catenin binding domain from a TCF transcription factor.

31. The method of claim 30, wherein the chimeric protein comprises amino acids 1-80 of the murine TCF-4 transcription factor.

32. The method of claim 26, wherein the chimeric protein comprises amino acids 1-147 of yeast GAL-4 protein and amino acids 1-80 of the murine TCF-4 transcription factor.

33. The method of claim 29, wherein the chimeric protein comprises a beta-catenin binding domain from a LEF transcription factor.

34. The method of claim 33, wherein the chimeric protein comprises amino acids 2-100 of the human LEF-1 transcription factor.
35. The method of claim 26, wherein the chimeric protein comprises amino acids 1-147 of yeast GAL-4 protein and amino acids 2-100 of the human LEF-1 transcription factor.
36. The method of claim 26, wherein said reporter gene is selected from chloramphenicol acetyl transferase, luciferase, beta-galactosidase, alkaline phosphatase, b-lactamase, horseradish peroxidase, green fluorescent protein and glutathione S-transferase.
37. The method of claim 26, wherein said reporter gene is luciferase.
38. The method of claim 26, wherein the first and second nucleic acid constructs are contained on a plasmid.
39. The method of claim 38, wherein the first and second nucleic acid constructs are contained on the same plasmid.
40. The method of claim 38, wherein the first and second nucleic acid constructs are contained on different plasmids.
41. The method of claim 26, wherein the first, second or first and second nucleic acid constructs are linear.
42. The method of claim 26, wherein the first, second or first and second nucleic acid constructs are circular.
43. The method of claim 26, wherein beta-catenin accumulation is induced by incubating the transfected cell with lithium chloride.
44. The method of claim 26, wherein the cell is selected from prokaryotic, yeast, plant, insect, worm, frog, fly, fish, mouse, rat, monkey, animal and mammalian cells.
45. The method of claim 44, wherein the cell is a Drosophila cell.
46. The method of claim 44, wherein the cell is a human cell.
47. The method of claim 26, wherein the cell is an epithelial cell.
48. The method of claim 26, wherein the cell is a HEK-293 cell.
49. The method of claim 26, wherein the level of transcription of the gene encoded for by the second nucleic acid construct is measured spectroscopically.
50. The method of claim 26, which is adaptable to a high-throughput format.

51. The method of claim 26, wherein the test compound is selected from polypeptides, nucleic acids, carbohydrates and small organic molecules.
52. The method of claim 26, wherein the test compound is a member of a library of compounds.
- 5 53. A method for affecting Wnt signal transduction comprising contacting a cell with an amount of a compound which modulates beta-catenin mediated transcriptional control, effective to change Wnt signal transduction.
54. A method for screening for compounds that can alleviate at least one symptom of a disease associated with abnormal cellular proliferation, comprising
- 10 (a) transfecting a cell with a first nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain from a transcription factor fused to a beta-catenin binding domain,
- (b) transfecting the cell with a second nucleic acid construct comprising coding sequences for a reporter gene located downstream from sequences
- 15 recognized by the DNA binding domain of said chimeric protein encoded for by the first nucleic acid construct,
- (c) inducing beta-catenin accumulation,
- (d) contacting said cell with a test compound,
- (e) measuring the level of transcription of the reporter gene, and
- 20 (f) comparing the level of expression of said reporter gene in the presence of said test compound and in the absence of said test compound,
- wherein step (c) may occur before, after or concurrently with step (d), and wherein a change in the level of reporter gene expression in the presence of said compound is indicative of a compound that modulates Wnt signaling
- 25 mediated cellular proliferation.
55. The method of claim 54, wherein the disease associated with abnormal cellular proliferation is cancer.
56. A nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain from a transcription factor fused to a beta-catenin binding domain.
- 30 57. The nucleic acid construct of claim 56, wherein the chimeric protein comprises a DNA binding domain from a yeast GAL-4 protein.

58. The nucleic acid construct of claim 57, wherein the chimeric protein comprises amino acids 1-147 of the yeast GAL-4 protein.
59. The nucleic acid construct of claim 56, wherein the chimeric protein comprises a beta-catenin binding domain from a member of the TCF/LEF family of transcription factors.
60. The nucleic acid construct of claim 59, wherein the chimeric protein comprises a beta-catenin binding domain from a TCF transcription factor.
61. The nucleic acid construct of claim 59, wherein the chimeric protein comprises amino acids 1-80 of the murine TCF-4 transcription factor.
- 10 62. The nucleic acid construct of claim 56, wherein the chimeric protein comprises amino acids 1-147 of yeast GAL-4 protein and amino acids 1-80 of the murine TCF-4 transcription factor.
63. The nucleic acid construct of claim 56, wherein the chimeric protein comprises a beta-catenin binding domain from a LEF transcription factor.
- 15 64. The nucleic acid construct of claim 63, wherein the chimeric protein comprises amino acids 2-100 of the human LEF-1 transcription factor.
65. The nucleic acid construct of claim 56, wherein the chimeric protein comprises amino acids 1-147 of yeast GAL-4 protein and amino acids 2-100 of the human LEF-1 transcription factor.
- 20 66. A chimeric protein comprising a DNA binding domain from a transcription factor fused to a beta-catenin binding domain.
67. The chimeric protein of claim 66 which comprises a DNA binding domain from a yeast GAL-4 protein.
68. The chimeric protein of claim 67 which comprises amino acids 1-147 of the yeast GAL-4 protein.
- 25 69. The chimeric protein of claim 66 which comprises a beta-catenin binding domain from a member of the TCF/LEF family of transcription factors.
70. The chimeric protein of claim 69 which comprises a beta-catenin binding domain from a TCF transcription factor.
- 30 71. The chimeric protein of claim 70 which comprises amino acids 1-80 of the murine TCF-4 transcription factor.

72. The chimeric protein of claim 66 which comprises amino acids 1-147 of yeast GAL-4 protein and amino acids 1-80 of the murine TCF-4 transcription factor.
73. The chimeric protein of claim 69 which comprises a beta-catenin binding domain from a LEF transcription factor.
- 5 74. The chimeric protein of claim 73 which comprises amino acids 2-100 of the human LEF-1 transcription factor.
75. The chimeric protein of claim 66 which comprises amino acids 1-147 of yeast GAL-4 protein and amino acids 2-100 of the human LEF-1 transcription factor.
- 10 76. A cultured cell expressing a chimeric protein comprising a DNA binding domain from a transcription factor fused to a beta-catenin binding domain.
77. The cultured cell of claim 76, wherein the chimeric protein comprises a DNA binding domain from a yeast GAL-4 protein.
78. The cultured cell of claim 77, wherein the chimeric protein comprises amino acids 1-147 of the yeast GAL-4 protein.
- 15 79. The cultured cell of claim 76, wherein the chimeric protein comprises a beta-catenin binding domain from a member of the TCF/LEF family of transcription factors.
80. The cultured cell of claim 79, wherein the chimeric protein comprises a beta-catenin binding domain from a TCF transcription factor.
- 20 81. The cultured cell of claim 80, wherein the chimeric protein comprises amino acids 1-80 of the murine TCF-4 transcription factor.
82. The cultured cell of claim 76, wherein the chimeric protein comprises amino acids 1-147 of yeast GAL-4 protein and amino acids 1-80 of the murine TCF-4 transcription factor.
- 25 83. The cultured cell of claim 79, wherein the chimeric protein comprises a beta-catenin binding domain from a LEF transcription factor.
84. The cultured cell of claim 83, wherein the chimeric protein comprises amino acids 2-100 of the human LEF-1 transcription factor.
- 30 85. The cultured cell of claim 76, wherein the chimeric protein comprises amino acids 1-147 of yeast GAL-4 protein and amino acids 2-100 of the human LEF-1 transcription factor.

86. The cultured cell of claim 76 which is a prokaryotic, yeast, plant, insect, worm, frog, fly, fish, mouse, rat, monkey, animal or mammalian cell.
87. The cultured cell of claim 86 which is a *Drosophila* cell.
88. The cultured cell of claim 86 which is a human cell.
- 5 89. The cultured cell of claim 76 which is an epithelial cell.
90. The cultured cell of claim 76 which is a HEK-293 cell.
91. A cultured cell comprising a nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain from a transcription factor fused to a beta-catenin binding domain.
- 10 92. The cultured cell of claim 91, wherein the chimeric protein comprises a DNA binding domain from a yeast GAL-4 protein.
93. The cultured cell of claim 92, wherein the chimeric protein comprises amino acids 1-147 of the yeast GAL-4 protein.
94. The cultured cell of claim 91, wherein the chimeric protein comprises a beta-
15 catenin binding domain from a member of the TCF/LEF family of transcription factors.
95. The cultured cell of claim 94, wherein the chimeric protein comprises a beta-catenin binding domain from a TCF transcription factor.
96. The cultured cell of claim 95, wherein the chimeric protein comprises amino
20 acids 1-80 of the murine TCF-4 transcription factor.
97. The cultured cell of claim 91, wherein the chimeric protein comprises amino acids 1-147 of yeast GAL-4 protein and amino acids 1-80 of the murine TCF-4 transcription factor.
98. The cultured cell of claim 94, wherein the chimeric protein comprises a beta-
25 catenin binding domain from a LEF transcription factor.
99. The cultured cell of claim 98, wherein the chimeric protein comprises amino acids 2-100 of the human LEF-1 transcription factor.
100. The cultured cell of claim 91, wherein the chimeric protein comprises amino acids 1-147 of yeast GAL-4 protein and amino acids 2-100 of the human LEF-1
30 transcription factor.
101. The cultured cell of claim 91 which is a prokaryotic, yeast, plant, insect, worm, frog, fly, fish, mouse, rat, monkey, animal or mammalian cell.

102. The cultured cell of claim 101 which is a *Drosophila* cell.
103. The cultured cell of claim 101 which is a human cell.
104. The cultured cell of claim 91 which is an epithelial cell.
105. The cultured cell of claim 91 which is a HEK-293 cell.
- 5 106. A cultured cell comprising
a first nucleic acid construct encoding for a chimeric protein comprising a DNA
binding domain from a transcription factor fused to a beta-catenin binding
domain, and
a second nucleic acid construct comprising coding sequences for a reporter gene
10 located downstream from sequences recognized by the DNA binding domain
of the chimeric protein encoded for by the first nucleic acid construct.
107. The cultured cell of claim 106, wherein the chimeric protein comprises a
DNA binding domain from a yeast GAL-4 protein.
108. The cultured cell of claim 107, wherein the chimeric protein comprises
15 amino acids 1-147 of the yeast GAL-4 protein.
109. The cultured cell of claim 106, wherein the chimeric protein comprises a
beta-catenin binding domain from a member of the TCF/LEF family of transcription
factors.
110. The cultured cell of claim 109, wherein the chimeric protein comprises a
20 beta-catenin binding domain from a TCF transcription factor.
111. The cultured cell of claim 110, wherein the chimeric protein comprises
amino acids 1-80 of the murine TCF-4 transcription factor.
112. The cultured cell of claim 106, wherein the chimeric protein comprises
amino acids 1-147 of yeast GAL-4 protein and amino acids 1-80 of the murine TCF-
25 4 transcription factor.
113. The cultured cell of claim 109, wherein the chimeric protein comprises a
beta-catenin binding domain from a LEF transcription factor.
114. The cultured cell of claim 113, wherein the chimeric protein comprises
amino acids 2-100 of the human LEF-1 transcription factor.
- 30 115. The cultured cell of claim 106, wherein the chimeric protein comprises
amino acids 1-147 of yeast GAL-4 protein and amino acids 2-100 of the human
LEF-1 transcription factor.

116. The cultured cell of claim 106, wherein the reporter gene is selected from chloramphenicol acetyl transferase, luciferase, beta-galactosidase, alkaline phosphatase, b-lactamase, horseradish peroxidase, green fluorescent protein, and glutathione S-transferase.
- 5 117. The cultured cell of claim 106, wherein the reporter gene is luciferase.
118. The cultured cell of claim 106, wherein the first and second nucleic acid constructs are contained on a plasmid.
119. The cultured cell of claim 118, wherein the first and second nucleic acid constructs are contained on the same plasmid.
- 10 120. The cultured cell of claim 118, wherein the first and second nucleic acid constructs are contained on different plasmids.
121. The cultured cell of claim 106, wherein the first, second or first and second nucleic acid constructs are linear.
122. The cultured cell of claim 106, wherein the first, second or first and second
15 nucleic acid constructs are circular.
123. The cultured cell of claim 106 which is a prokaryotic, yeast, plant, insect, worm, frog, fly, fish, mouse, rat, monkey, animal, or mammalian cell.
124. The cultured cell of claim 123 which is a *Drosophila* cell.
125. The cultured cell of claim 123 which is a human cell.
- 20 126. The cultured cell of claim 106 which is an epithelial cell.
127. The cultured cell of claim 106 which is a HEK-293 cell.
128. A method for inducing death in a cell containing a Wnt signaling pathway, comprising:
- 25 (a) transfecting a cell with a first nucleic acid construct encoding for a chimeric protein comprising a DNA binding domain from a transcription factor fused to a beta-catenin binding domain, and
- (b) transfecting the cell with a second nucleic acid construct comprising coding sequences for a toxin gene located downstream from sequences recognized by the DNA binding domain of said chimeric protein encoded for by the first
30 nucleic acid construct,
- wherein the toxin gene is expressed in response to the presence of beta-catenin and is capable of inducing cell death.

129. The method of claim 128, wherein the toxin gene is selected from diphtheria toxin, ricin, cytokine genes, tumor suppressor genes, DNA sequences that yield anti-sense RNA to oncogenes, and genes that induce apoptosis.

130. The method of claim 129, wherein the toxin gene is p53.

5

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LITHIUM CHLORIDE DOSE RESPONSE (LEF)

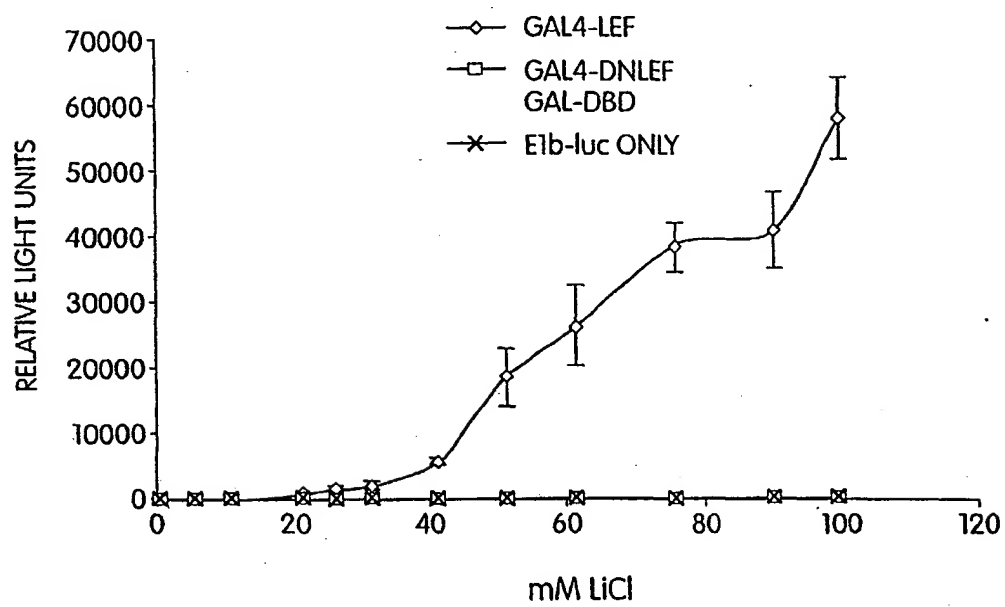


Fig. 1

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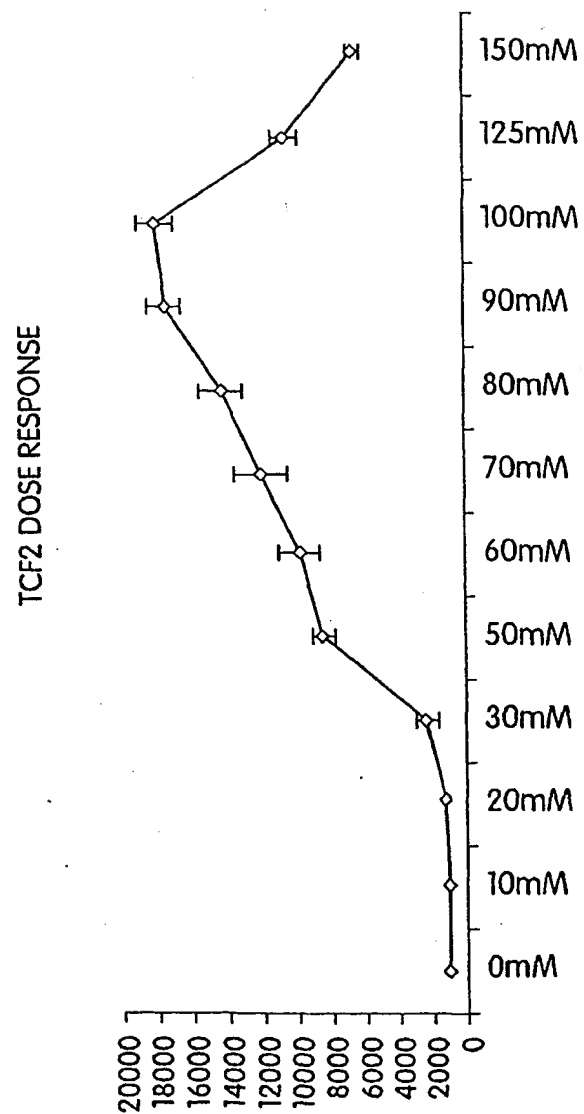


Fig. 2

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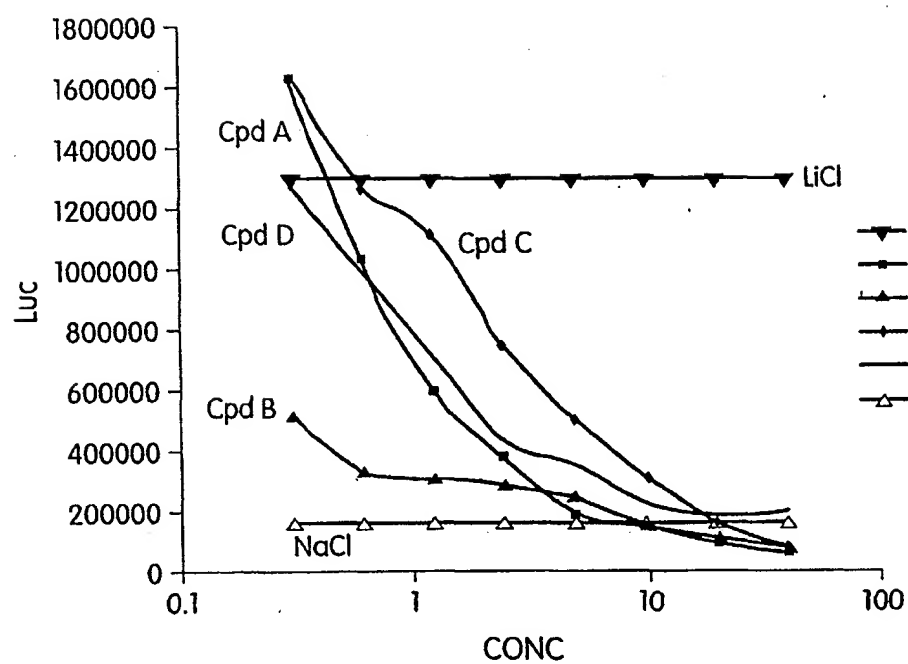


Fig. 3

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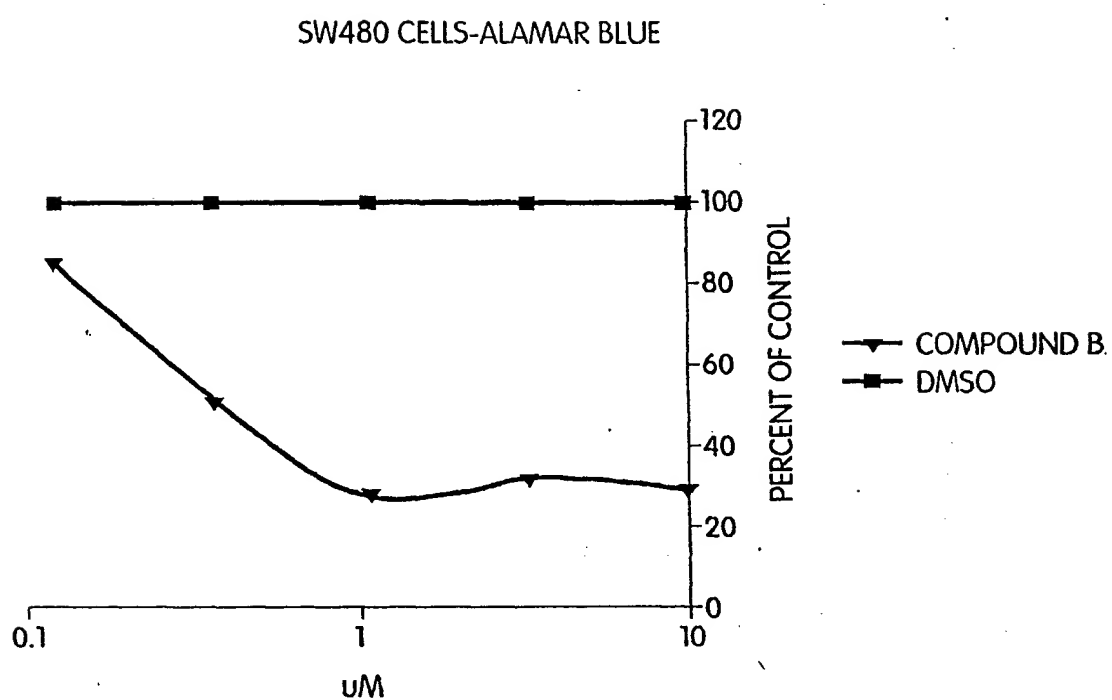


Fig. 4

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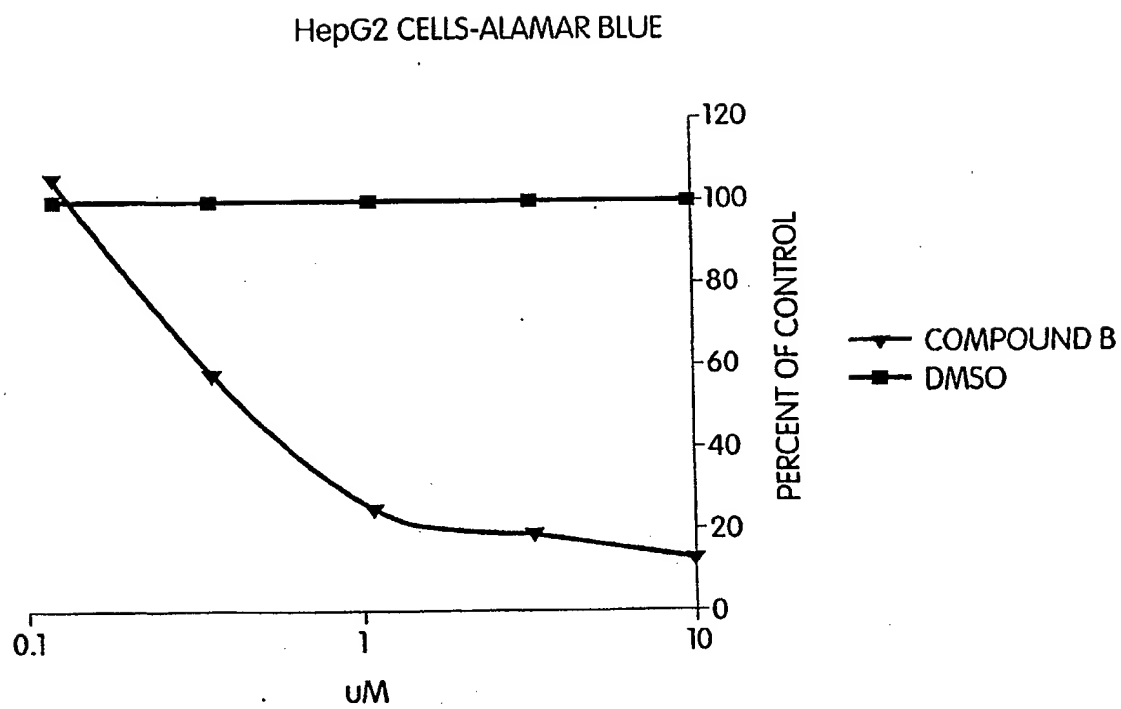


Fig. 5

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	Glu Gln Leu Phe Leu Leu Ile Phe Pro Arg Glu Asp Leu Asp Met Ile		
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	Leu Lys Met Asp Ser Leu Gln Asp Ile Lys Ala Leu Leu Thr Gly Leu		
	85	90	95
15	Phe Val Gln Asp Asn Val Asn Lys Asp Ala Val Thr Asp Arg Leu Ala		
	100	105	110
	Ser Val Glu Thr Asp Met Pro Leu Thr Leu Arg Gln His Arg Ile Ser		
	115	120	125
20	Ala Thr Ser Ser Ser Glu Glu Ser Ser Asn Lys Gly Gln Arg Gln Leu		
	130	135	140
	Thr Val Ser Ile Asp Ser Ala Ala His His Asp Asn Ser Thr Ile Pro		
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	Leu Asp Phe Met Pro Arg Asp Ala Leu His Gly Phe Asp Trp Ser Glu		
	165	170	175
30	Glu Asp Asp Met Ser Asp Gly Leu Pro Phe Leu Lys Thr Asp Pro Asn		
	180	185	190
	Asn Asn Gly Phe Phe Gly Asp Gly Ser Leu Leu Cys Ile Leu Arg Ser		
	195	200	205
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	Pro Thr Met Ile Thr Asp Arg Tyr Thr Leu Ala Ser Arg Ser Thr Thr		
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	Ser Arg Leu Leu Gln Ser Tyr Leu Asn Asn Phe His Pro Tyr Cys Pro		
	245	250	255
45	Ile Val His Ser Pro Thr Leu Met Met Leu Tyr Asn Asn Gln Ile Glu		
	260	265	270
	Ile Ala Ser Lys Asp Gln Trp Gln Ile Leu Phe Asn Cys Ile Leu Ala		
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			595					600					605				
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		610					615					620					
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 10 Asn Ile Ser Val Lys Tyr Val Ser Pro Gly Ser Val Gly Pro Ser Pro
 690 695 700
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 725 730 735
 20 Thr Pro Ser His Arg Ser Val Thr Pro Phe Leu Gly Gln Gln Gln Gln
 740 745 750
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 40 Phe Gly Ile Thr Thr Gly Met Phe Asn Thr Thr Thr Met Asp Asp Val
 850 855 860
 Tyr Asn Tyr Leu Phe Asp Asp Glu Asp Thr Pro Pro Asn Pro Lys Lys
 865 870 875 880
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